

Quality of Thrombin Produced From the Patient's Own Plasma Using the TPD™, a New Thrombin-Processing Device

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Abstract: Thrombin derived from bovine sources commonly is used to arrest bleeding during surgical procedures. However, complications such as postoperative hemorrhage can occur because of the development of cross-reactive anti-bovine antibodies that inhibit human coagulation factor V. It would thus be advantageous to develop techniques to generate human thrombin. This study evaluated thrombin produced from human plasma using a new Thrombin-Processing Device (TPD™). Plasma was introduced into the TPD, mixed with an ethanol/CaCl₂ reagent, incubated for 1 h, and the harvested thrombin was assayed for activity and the ability to activate platelets by in vitro assays. TPD-produced thrombin activity was found to be

51.8 ± 12.4 IU/mL (*n* = 145). TPD-produced thrombin also stimulated P-selectin (CD62) expression (83 ± 13% of the platelet population) and Annexin V binding (10.3 ± 2% of the platelet population) on platelets in a similar fashion to commercial thrombin (P-selectin expression: 88 ± 3%; Annexin-V binding: 11.4 ± 3%). Compared with CaCl₂ and batroxobin, TPD-produced thrombin had a significantly greater ability to activate platelets. TPD-produced thrombin from human plasma has consistent activity and significantly activates platelets and, thus, may have attractive applications such as the production of autologous thrombin for surgical patients. **Keywords:** human thrombin, platelet activation, autologous. *JECT. 2005;37:196–200*

Thrombin is a serine protease important for the mediation and the regulation of the coagulation cascade. The production of thrombin is initiated on the surface of endothelial cells and activated platelets by the formation of the prothrombinase complex. This complex is composed of platelet phospholipids, Ca²⁺, and coagulation factors Va and Xa, and it proteolytically cleaves prothrombin to thrombin (1). Thrombin is not only critical for the enhancement of coagulation, but it also plays an important role in its control. For example, combined with thrombomodulin present on the endothelial cell surface, thrombin converts protein C to protein Ca. Together with protein S, protein Ca can degrade factors Va and VIIIa, thus limiting the activity of the coagulation cascade. These events are continually ongoing (e.g., at the site of injury) and are

responsible for not only reducing blood flow but also promoting wound healing and angiogenesis (2).

Thrombin has been used in surgical procedures as a means of reducing wound bleeding for decades. In 1977, Jasani reported that 15 patients treated with topical thrombin after abdominal surgery exhibited significantly fewer hematomas compared with the control group not receiving treatment (3). This finding was later confirmed by Hashemi et al., who showed that heparinized patients receiving treatment with topical thrombin had a reduced rate of hematoma formation (4). Furthermore, thrombin also has been used successfully for the control of bleeding during skin grafting in burn patients (5) and in cardiac surgery (6), among other disciplines.

The combination of thrombin with concentrated fibrinogen (cryoprecipitate) and platelets is also a common clinical practice to prevent bleeding and to promote wound healing (7). For example, fibrin sealants (thrombin combined with fibrinogen) are used in a variety of surgical procedures and have been shown to reduce bleeding (8), seroma formation, and drain usage (9). However, thrombin, in combination with platelets is used mainly in ortho-

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pedic (10), oral, and maxillofacial surgery (11) for enhancement of bone growth attributable to the increased level of tissue growth factors present in the platelets (12).

To date, thrombin used in surgery primarily is produced from bovine plasma sources. A major problem with the use of bovine thrombin is that it can generate the formation of anti-bovine thrombin antibodies (13). These antibodies have been shown to cross-react with the patient's coagulation Factor V (FV) and cause a range of symptoms, including severe and life-threatening bleeding (13). In 2001, Ortel et al. reported on the risks and prevalence of these adverse reactions and investigated both the formation of antibodies against bovine thrombin and the cross-reactivity against the patient's FV (14). They found elevated antibody levels against human coagulation factors in 20% of the patients and concluded that bovine thrombin preparations were highly immunogenic and associated with increased risk of adverse outcomes and that re-exposure should be avoided. In addition, the outbreak of bovine spongiform encephalopathy and its eventual linkage to variant Creutzfeldt–Jacob disease in humans (15) has elevated the awareness of substituting bovine products with human, whenever possible. These data suggested that bovine thrombin, although able to reduce bleeding, caused significant adverse events as the result of antibody formation and transmission of disease. To prevent these events, a relatively simple method to produce human thrombin with similar haemostatic properties to commercially available thrombin would be desirable.

We developed the Thrombin-Processing Device (TPD™; Thermogenesis Corp, Rancho Cordova, CA), which consists of two parts: a tubular reaction chamber containing a negative surface charge required for initiation of the formation of thrombin and a thrombin reagent consisting of calcium chloride and ethanol. Plasma and thrombin reagent was added to the TPD reaction chamber and the contents were mixed and incubated at room temperature. The TPD was then agitated to break any formed fibrinogen clots and the produced thrombin was ready for harvest.

Table 1. Final concentration of agonists used for the activation of platelets.

| Thrombin (TPD produced and commercial) | CaCl ₂ | Batroxobin* |
|--|-------------------|-------------|
| 0.01 IU/mL | 1 mM | 50 µL |
| 0.1 IU/mL | 10 mM | 100 µL |
| 1 IU/mL | 20 mM | 150 µL |
| 10 IU/mL | 100 mM | |

*Batroxobin was diluted according to the manufacturer's instructions to a concentration giving clotting in 16–22 sec.

The aim of this study was to assess the activity and platelet-activating ability of the thrombin produced by the TPD and compare it with commercially available thrombin, Ca²⁺, or batroxobin. Our results show that the TPD-produced thrombin is stable over the course of 6 h and has a similar ability to activate platelets compared with commercial thrombin. The results suggest that the TPD can be used for production of human thrombin from a single donor for autologous use during surgery.

MATERIALS AND METHODS

The Thrombin-Processing Device

The TPD consists of two parts: (1) a tubular reaction chamber containing ceramic beads providing a negative surface charge required for initiation of the formation of thrombin and (2) a thrombin reagent consisting of calcium chloride and ethanol (final concentration 7.2 mM and 19%, respectively, Thermogenesis Corp).

Preparation of Platelet-Poor and Platelet-Rich Plasma (PRP)

Whole blood, collected in 3.8% citrate solution, was obtained from healthy volunteer blood donors. PRP was separated from collected whole blood by centrifugation at 1000g for 20 min. The supernatant plasma was removed and used in the TPD. For a source of platelets, PRP was prepared from collected whole blood by low speed centrifugation (210g for 15 min).

Preparation of Thrombin

Ten milliliters of PRP plasma and 4 mL of thrombin reagent were added to the TPD reaction chamber. The contents were mixed and incubated at room temperature for 50 min. The TPD was agitated to break any formed fibrinogen clots and incubated for an additional 10 min. The produced thrombin was harvested, aliquoted in 3-mL samples, and frozen at –80°C until analysis.

Thrombin Activity

Thrombin activity was analyzed using a modified method of Clauss (16). Briefly, 100 µL of a 2.5 mg/mL prewarmed (37°C) solution of fibrinogen (Sigma, St. Louis, MO), solution was added to 50 µL of a pre-warmed (37°C) thrombin sample. The time required for clot formation was recorded using a MLA 900 coagulation device (Diamond Diagnostics, Holliston, MA). Quantitation of thrombin activity in the samples was determined by correlating the time to clot formation to a standard curve generated with titrations of commercial bovine thrombin (Jones Pharma Inc., Bristol, TN) standard curve to quantitate activity. Because each time point on the standard curve corresponds to the thrombin activity needed to clot a standard concentration of fibrinogen, the thrombin ac-

tivity in an unknown sample can be determined by the time to clotformation.

Assessment of the Stability

The stability of the TPD-produced thrombin was assessed by storing 3 mL of thrombin at either 4°C (between ice packs) for 4 h with an additional 2 h at 37°C or at 37°C for 6 h. Thrombin activity was analyzed at time 0 (after preparation) and after 4 and 6 h of storage.

Platelet Activation

Activation of platelets was performed by adding titrations of either TPD-produced thrombin, CaCl₂, commercial thrombin (Ortho, San Jose, CA), or batroxobin (Dade Behring, Marburg, Germany) in 10 to 90 µL of PRP. To prevent clot formation during platelet activation, the peptide Gly-Pro-Arg-Pro (GPRP, Sigma) was added at a final concentration of 2.4 mM. This tetrapeptide binds with a high affinity to fibrinogen and inhibits fibrin polymerization. The samples were incubated in a 37°C waterbath for 10 min. After incubation, 5 µL of PRP was removed and the remaining PRP was fixed using paraformaldehyde (0.5% final concentration) for 10 min. The fixation was stopped by the addition of 1 mL of phosphate-buffered saline. Annexin-V binding was assessed on the nonfixed PRP preparation and P-selectin (CD62) expression was evaluated on the fixed PRP by flowcytometry. Briefly, 50 µL of nonfixed or fixed PRP were both stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD41 antibody (Ortho) for 30 min at room temperature and washed once. The nonfixed and fixed samples were then stained with either phycoerythrin (PE)-conjugated Annexin V (Becton-Dicinson) or PE-conjugated anti-CD62 (Becton-Dicinson), respectively, for 30 min at room temperature, in the dark, and then fixed using paraformaldehyde (0.5% final concentration). The platelets were acquired on a flow cytometer (Facs-Scan, Becton Dicinson) operating with an argon laser at 15 mW. Ten thousand events were acquired and analyzed using Cellquest software.

Time course experiments were performed at set concentrations of the agonists (final concentration 1 IU/mL of TPD produced thrombin and commercial thrombin, 10 mM CaCl₂, and batroxobin in a concentration causing clot formation in 16–22 s, according to the manufacturer).

Statistical Analysis

All results are presented as mean and standard deviation, unless otherwise stated. Significance between groups was assessed by Student's *t* test.

RESULTS

Thrombin Activity in TPD-Produced Thrombin

Thrombin activity in TPD produced thrombin was analyzed in 145 samples and found to be 51.8 ± 12.4 IU/mL.

Stability of TPD-Produced Thrombin

The stability of TPD-produced thrombin was tested after 4 and 6 h of storage at either 4°C or at 37°C (n = 10/group). After preparation, the thrombin activity was found to be 47.0 ± 8 IU/mL. There was no significant difference in the thrombin activity immediately after preparation or after 4 or 6 h of storage at 37°C (39 ± 7 IU/mL versus 38 ± 6 IU/mL, respectively) or after 4 h at 4°C (39 ± 11 IU/ml) and subsequent 2 h at 37°C (36 ± 11 IU/mL).

Dose Response

To test the dose–response of the activating agents, different concentrations of the agonists were added to the PRP and the platelets were activated for 10 min (Table 1). No difference was found in the expression of P-selectin or Annexin V binding between commercial thrombin and TPD-produced thrombin (Figure 1). Increasing the thrombin activity showed a dose-dependent increase, with a plateau at 1 IU/mL. A final concentration of 10 mM CaCl₂ stimulated the platelets to express approximately 60% of P-selectin and increasing to 20 mM only increased the activation slightly (Figure 2). Adding 100 mM of CaCl₂ overcame the inhibition of the addition of GPRP and clot formation occurred (Figure 2). Stimulating platelets with batroxobin did not cause activation of the platelets as measured by P-selectin expression.

Time of Activation

To test the ability of the agonists to stimulate platelets, platelets were activated for various times periods. Using either TPD-produced thrombin and commercial thrombin, the platelets expressed more than 80% of their P-

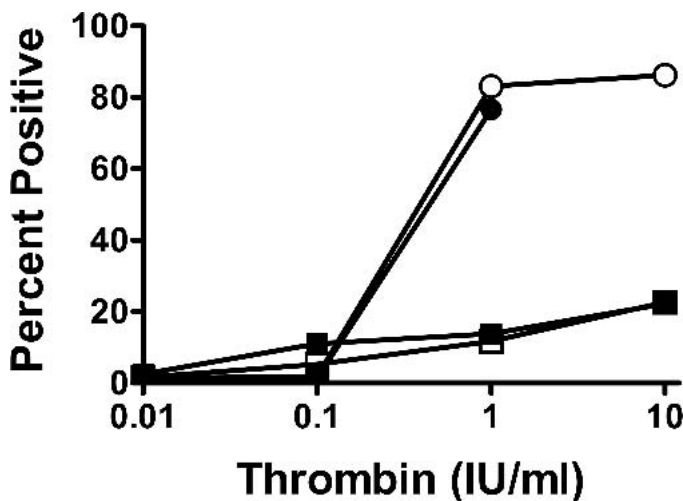


Figure 1. Expression of CD62 (P-selectin) and binding of Annexin V, expressed as percent positive for corresponding marker, in platelets stimulated with commercial thrombin or TPD-produced thrombin at different concentrations. TPD-produced thrombin: ●, CD62 expression; ■, Annexin V. Commercial thrombin: ○, CD62 expression; □, Annexin V.

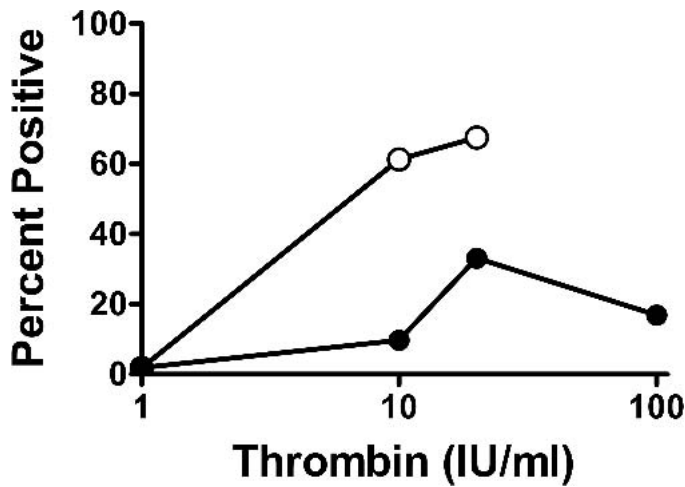


Figure 2. Expression of CD62 (P-selectin) and binding of Annexin V, expressed as percent positive for corresponding marker, in platelets stimulated with CaCl₂ at different concentrations. ○, CD62 expression; ●, Annexin V.

selectin by 30 s of incubation (Figure 3). However, using CaCl₂, maximal platelet activation required significantly more time (Figure 3). After 5 min, the expression of P-selectin was less than 20% and only increased to 60% by 10 s (Figure 3). In the samples incubated for 30 s, clot formation occurred, making the analysis impossible. Batroxobin did not activate the platelets in this setting (Figure 3) and no expression of P-selectin over the baseline expression was observed in any of the samples.

The platelet's ability of binding Annexin V after stimulation for different time periods also was investigated. Using TPD-produced thrombin or commercial thrombin, approximately 10% binding was detected after 30 s, which

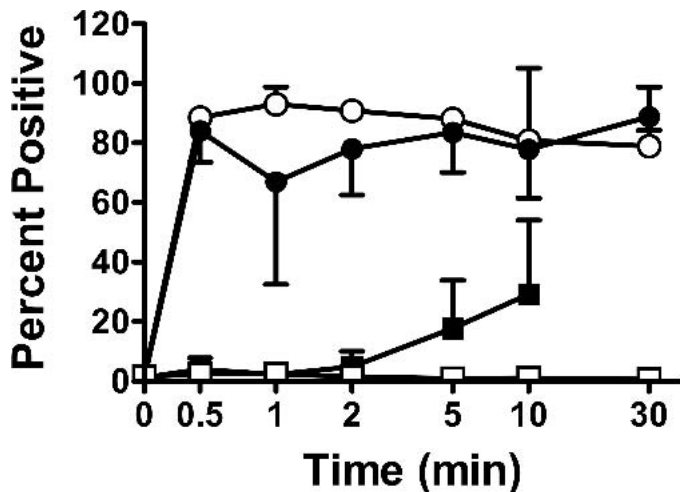


Figure 3. Platelet expression of CD62 (P-selectin) after stimulation with TPD-produced thrombin (1 IU/mL), commercial thrombin (1 IU/mL), Ca²⁺ (10mM), and batroxobin (diluted to produce a clot in 16–22 s) after different times of incubation. The data are expressed as percent positive for corresponding marker. ●, TPD-produced thrombin; ○, commercial thrombin; ■, CaCl₂; □, batroxobin.

did not change significantly over the course of the remaining incubation period (Figure 4). There was no significant difference between commercial thrombin and TPD produce thrombin (Figure 4).

Stimulating platelets with CaCl₂ resulted in lower binding of Annexin V after 30 s compared with commercial thrombin or TPD-produced thrombin. Although there appeared to be a trend of lower binding of Annexin V using CaCl₂ the difference did not reach significance at any of the time points after 1 of incubation (Figure 4). Batroxobin did not bind Annexin V in this setting (Figure 4).

DISCUSSION

To date, thrombin used for surgery primarily is derived from bovine sources, which can lead to significant adverse reactions as a result of the development of cross-reactive antibodies that reacts with human coagulation factor V (13). Methods to generate human thrombin, preferably from the patient's own plasma, are important to alleviate these types of adverse events. The TPD has been developed for the production of human thrombin from single donor plasma. In the presence of the negatively charged surface (the ceramic beads) and plasma, the thrombin reagent (CaCl₂ and ethanol) initiates the formation of the prothrombinase complex and subsequent promotion of active thrombin. This study has shown that thrombin produced by the TPD has a thrombin activity of 51 IU/mL and is stable over the course of 6 h. Commercial thrombin used in surgery commonly has a higher activity (500 or 1000 IU/mL). However, it has been shown that thrombin with an activity of 50 IU/mL gives a superior adhesion to the wound site when combined with fibrino-

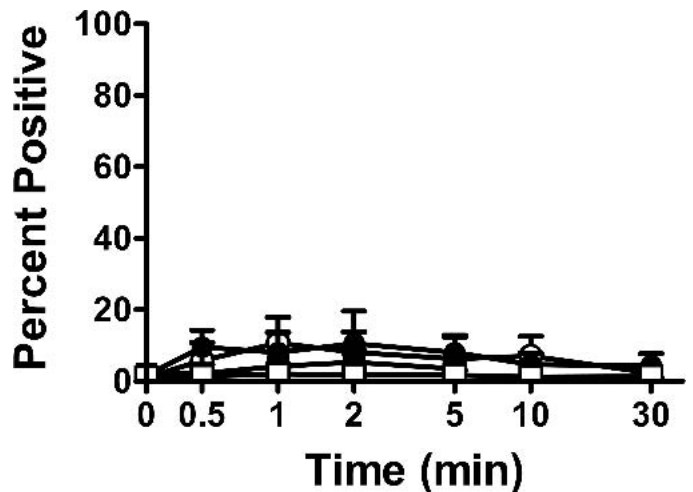


Figure 4. Binding of Annexin V to platelets stimulated with TPD-produced thrombin (1 IU/mL), commercial thrombin (1 IU/mL), Ca²⁺ (10 mM) and batroxobin (diluted to give a clot in 16–22 s) after different times of incubation. The data are expressed as percent positive for corresponding marker. ●, TPD-produced thrombin; ○, commercial thrombin; ■, CaCl₂; □, batroxobin.

gen concentrate compared with thrombin with an activity of 500 IU/mL (17).

To test the TPD produced thrombin's ability to activate platelets, we examined platelet P-selectin (also known as CD62) expression and Annexin V binding. P-selectin is an adhesion molecule expressed on the internal alpha-granule membrane of resting platelets (18). When platelets are activated by thrombin, the alpha-granule membrane fuses with the external membrane and all contents are expressed or released to the external milieu. P-selectin expression is responsible for activated platelets to adhere to subendothelium to eventually arrest bleeding and has in vitro been shown to be one of the best markers of platelet activation (19). However, the outer membrane of activated platelets also expresses high levels of negatively charged phosphatidylserine molecules and these can be identified by their ability to bind Annexin V. Once phosphatidylserine has been exposed at the cell surface, it exhibits procoagulant and proinflammatory activities and thus, Annexin V binding is a good measure of the coagulability of the platelets (20). Our results show that the coagulability is preserved in the platelets exposed to TPD-produced thrombin.

The TPD-produced thrombin and commercial thrombin-activated platelets to the same extent when used at the same concentration. There was no significant difference found in the expression of P-selectin or binding of Annexin V between the two agents in regards to concentration needed for activation or time to activation. Using commercial thrombin or TPD produced thrombin, platelets expressed approximately 80% of their total P-selectin after 30 s (Figure 3). In comparison, CaCl₂ activated the platelets, but the activation was much slower. After 10 s, only 60% of the platelets were activated. However, batroxobin, was found to not activate the platelets at all. This confirms what has been shown earlier by Francischetti et al. (21), and the data suggest that TPD-produced thrombin has similar properties compared with commercial thrombin.

In conclusion, human thrombin produced by the TPD has a stable activity and induces platelet activation in a similar manner to commercially available bovine thrombin. Collectively, these data suggest that TPD-produced thrombin may have an advantage in that it can be used to produce thrombin in an autologous setting for surgical patients.

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