

## Expression, refolding, and activation of the catalytic domain of human blood coagulation factor XII

Jixiu Shan, Marilyn Baguion, Li Zheng, and Ramaswamy Krishnamoorthi\*

*Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA*

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### Abstract

Human blood coagulation factor XII (FXII; 80 kDa) contains a C-terminal serine protease zymogen domain, which becomes activated upon contacting a negative surface. Activated FXII ( $\alpha$ FXIIa) brings about reciprocal activation of FXII and kallikrein that by further hydrolysis produces the free catalytic domain ( $\beta$ FXIIa; 28 kDa). Increased levels of  $\alpha$ FXIIa are associated with coronary heart disease, sepsis, and diabetes. Biophysical investigation of the structural basis of activation, substrate specificity, and regulation of FXII requires an efficient bacterial system for producing the wild-type and mutant recombinant proteins. Here, the cDNA of the zymogen domain of FXII ( $\beta$ FXII) was cloned into the pET-28a(+) vector and the plasmid was transformed into *Escherichia coli* strain BL21 (DE3) and overexpressed. The multi-disulfide, recombinant protein, His(6)- $\beta$ FXII (r $\beta$ FXII), expressed as an inclusion body, was purified by means of a Ni<sup>2+</sup>-charged resin. The matrix-bound r $\beta$ FXII was subjected to refolding with the glutathione redox system and activated by the in vivo activator, kallikrein. The active form, r $\beta$ FXIIa, obtained in milligram quantities, exhibited similar structural and comparable functional properties relative to human  $\beta$ FXIIa, as indicated by circular dichroism spectroscopy and kinetics of substrate hydrolysis. Thermodynamics of enzyme:inhibitor complex formation, including the expected 1:1 stoichiometry, was determined for r $\beta$ FXIIa by isothermal calorimetric titration with a specific recombinant protein inhibitor, *Cucurbita maxima* trypsin inhibitor-V (rCMTI-V; 7 kDa).

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The blood coagulation cascade along the intrinsic pathway is initiated by the single-chain zymogen, factor XII (FXII; ~80 kDa), also called Hageman factor, when it is converted into the two-chain serine protease  $\alpha$ FXIIa by the hydrolysis of its Arg<sup>373</sup>-Val<sup>374</sup> peptide bond [1]. Anionic components of subendothelial basement membrane, phospholipids, neutrophils, and cell surfaces are suggested to be activating physiological surfaces.  $\alpha$ FXIIa in turn activates FXII (autoactivation) and converts prekallikrein to kallikrein, another serine protease, and FXI to FXI $\alpha$ . Kallikrein produces more  $\alpha$ FXIIa and further hydrolyzed products, including  $\beta$ FXIIa, which is a 28 kDa domain responsible for the enzymatic activity of FXII [2]. Although deficiency of

FXII in individuals does not cause bleeding [2], increased levels of  $\alpha$ FXIIa are linked to certain pathological states, such as coronary heart disease [3], sepsis [4], and diabetes [5]. Understanding the structural basis of activation, substrate specificity, and regulation of FXII is of importance for developing strategies to prevent FXII activation.

No three-dimensional structure has yet been reported for FXII,  $\alpha$ FXIIa, or  $\beta$ FXIIa. Computer-modeling shows that  $\beta$ FXIIa bears a strong three-dimensional structural homology to the pancreatic serine proteases, trypsin, chymotrypsin, and elastase [6]. The catalytic triad His<sup>57</sup>-Asp<sup>102</sup>-Ser<sup>214</sup> of trypsin can be aligned with the corresponding residues of  $\beta$ FXIIa (His<sup>395</sup>, Asp<sup>442</sup>, and Ser<sup>544</sup> in FXII). Previous studies have identified the functional roles of several domains of FXII [7–9] and the importance of Zn(II) as an effector in activation

\* Corresponding author. Fax: 1-785-532-7278.

E-mail address: [krish@ksu.edu](mailto:krish@ksu.edu) (R. Krishnamoorthi).

[10–14]. However, detailed structural studies of the zymogen and enzyme forms of FXII or its catalytic domain are yet to be reported, perhaps because of nonavailability of recombinant proteins in needed quantities. We have isolated and determined the three-dimensional structure and dynamic properties of a specific inhibitor of human  $\beta$ FXIIa *Cucurbita maxima* trypsin-inhibitor-V [CMTI-V; 7 kDa] [15–18]. Several genetically engineered point mutants of CMTI-V, such as T43S, T43N, K44R, R47Q, R50Q, and R52Q, show significantly altered inhibitory properties toward  $\beta$ FXIIa only, *but not trypsin* ([19]; Wen et al., unpublished results). Thermodynamic studies of binding of inhibitors to their cognate enzymes provide macroscopic details about interactions at the enzyme:inhibitor interface, and often, in conjunction with mutant inhibitors and mutant enzymes, provide a powerful tool to delineate the structural basis of enzyme substrate specificity [20–25].

Herein, we report the cDNA cloning and overexpression of human  $\beta$ FXII, the zymogen form of the catalytic domain of human FXII. The expressed recombinant His(6)- $\beta$ FXII (r $\beta$ FXII) was purified in a single step, successfully refolded and activated by kallikrein, and shown to have similar structural and functional properties as the natural enzyme, human  $\beta$ FXIIa, by circular dichroism (CD) spectroscopy and kinetics of substrate hydrolysis. Further, the thermodynamics, including the expected 1:1 stoichiometry, of binding of recombinant CMTI-V (rCMTI-V) by activated r $\beta$ FXII (r $\beta$ FXIIa) was determined by isothermal titration calorimetry (ITC).

## Materials and methods

### Materials

The cDNA of human  $\beta$ FXII, the catalytic domain of FXII, which was inserted in pSinHis B (Invitrogen Corporation), was a gift from Dr. Lisa Wen (Western Illinois University). pET28a(+) was used as the expression vector and *Escherichia coli* strains JM109 and BL21 (DE3) were used as the host; these were products of Novagen. PCR and ligation reagents were purchased from Promega. Reduced and oxidized forms of glutathione (GSH and GSSG, respectively), DL-dithiothreitol (DTT), *n*-dodecyl  $\beta$ -D-maltoside (DM), imidazole (1,3-diaza-2,4-cyclopentadiene), bovine trypsin, and lysozyme were from Sigma. Human plasma coagulation factor  $\beta$ FXIIa, human plasma kallikrein, and iso-propylthio- $\beta$ -galactopyranoside (IPTG) were from CalBiochem. His-Bind Resin was from Novagen. The chromogenic substrates S2302 (D-Pro-L-Phe-L-Arg-p-nitroanilide) and S2222 (Ile-Glu-Gly-Arg-p-nitroanilide) were purchased from Chromogenix. rCMTI-V was prepared, as described previously [19].

### Construction of *E. coli* expression plasmids

The DNA encoding human  $\beta$ FXII was amplified by the polymerase chain reaction (PCR). pSinHis B which contained the cDNA of human  $\beta$ FXII was used as the template. PCR primers were: 5'-CG GAA TTC (*EcoRI*) CTG ACC AGG AAC GGC CCA-3' and 5'-CCC AAG CTT (*HindIII*) GGG CCC TCA GGA AAC GGT-3'. The PCR products were isolated and double digested with *EcoRI* and *HindIII*, then ligated into the expression vector, pET28a(+), which had been digested with the same endonucleases, and transformed into competent *E. coli*. The sequence and the orientation of the insert were confirmed by DNA sequencing.

### Expression

Transformed *E. coli* strain BL21 (DE3) cells were first grown on Luria–Bertani (LB) medium plates supplemented with 50  $\mu$ g/ml kanamycin (Sigma). Single colonies were inoculated into 12 ml LB medium containing 50  $\mu$ g/ml kanamycin. After overnight growth, this culture was transferred to a 2-liter flask containing 500 ml M9ZB medium supplemented with 50  $\mu$ g/ml kanamycin. Cells were grown with vigorous shaking (300 rpm) to a density of  $OD_{600} = 0.8$ –1.0, then the expression of recombinant protein was induced with 1 mM IPTG, and the incubation was continued overnight at room temperature.

### Purification of recombinant His(6)- $\beta$ FXII (r $\beta$ FXII)

Cells were collected by centrifugation at 7500g for 15 min, resuspended with a buffer of 50 mM Tris-HCl, pH 8.0, and treated with 150–200  $\mu$ g/ml lysozyme at room temperature for 30 min, followed by sonication. The lysate was centrifuged at 27,000g, 4°C. At this point, both the pellet and supernatant were analyzed for the solubility of the recombinant protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; [26]). The pellet was suspended in a buffer of 20 mM Tris-HCl, pH 7.9, with 5 mM imidazole, 500 mM NaCl, and 8 M urea. After being stirred for 1 h at room temperature, the sample was centrifuged at 30,000g, 4°C. The supernatant was transferred into a tube containing Ni<sup>2+</sup>-charged His-Bind Resin and the mixture was spun on a Rotamix for 30 min. The recombinant protein-bound resin was washed in a column with a wash buffer of 20 mM Tris-HCl, pH 7.9, with 35 mM imidazole, 500 mM NaCl, and 8 M urea. The washing was monitored by measuring absorbance at 595 nm of the eluate with the wash buffer as control and the purity of r $\beta$ FXII was checked by SDS–PAGE.

### Refolding of r $\beta$ FXII

The expressed recombinant protein was directly refolded, while it was immobilized on the His-Bind Resin, as follows: The washed His-resin with the bound r $\beta$ FXII was added dropwise, under stirring, into a reduction buffer of 100 mM Tris-HCl, pH 8.4, 5 mM imidazole, 5 mM DTT, and 6 M urea at 4°C; at least 20 ml buffer per milligram of the protein was needed. Stirring was continued for 1 h and the reduction buffer was replaced with an oxidation buffer of 100 mM Tris-HCl, pH 8.0, 5 mM imidazole, 0.78 mM GSSG, 7.8 mM GSH, and 6 M urea. The concentration of the protein in this buffer was 0.1 mg/ml. All the sample was transferred into a column with a magnetic bar at the bottom and diluted with two kinds of dilution buffers successively, the first one being 100 mM Tris-HCl, pH 8.0, 5 mM imidazole, 0.78 mM GSSG, and 7.8 mM GSH, and the second one being 100 mM Tris-HCl, pH 8.0, 5 mM imidazole. The rate of dilution was kept under 0.25 ml/min. If and when the His-resin aggregated at the bottom, the column was mounted on a magnetic stirrer and dilution continued under stirring. The refolded r $\beta$ FXII was eluted from the resin with the elution buffer of 100 mM Tris-HCl, pH 8.0, 1 M imidazole, and 0.01% (w/v) DM; or, it was directly used for activation.

### Activation of refolded r $\beta$ FXII

The reported procedure [10] was modified: the His-resin, which bound refolded His(6)- $\beta$ FXII, was washed with an activation buffer of 100 mM Hepes, pH 7.4, 100 mM NaCl, 100  $\mu$ M ZnCl<sub>2</sub>, and 0.1% (w/v) PEG 8000. The activation buffer was retained in the column at a ratio 1:1 (v/v) with the resin. Kallikrein was added to refolded r $\beta$ FXII at a molar ratio of 0.0075:1 and the mixture was spun on the Rotamix for 45 min at room temperature. After incubation, the reaction mixture was washed with a buffer of 100 mM Tris-HCl, pH 8.0, 5 mM imidazole, and 0.01% (w/v) DM until no amidolytic activity of kallikrein with the substrate S2302 was detected in the washing. Activated His(6)- $\beta$ FXII (r $\beta$ FXIIa) was eluted from the resin with the elution buffer of 100 mM Tris-HCl, pH 8.0, 1 M imidazole and amidolytic activity of r $\beta$ FXIIa was measured. A blank was done by mixing the activation buffer with the His-resin that had no r $\beta$ FXII bound to it, followed by kallikrein treatment and washing. The washing was checked for amidolytic activity with S2302.

### Circular dichroism measurements

Purified r $\beta$ FXII which was still bound to the HisBind Resin was directly used for the refolding step. Refolded r $\beta$ FXII was, before or after activation by kallikrein, eluted from the column by a solution containing 1 M

imidazole. For CD measurements, the protein solution was prepared after dialyzing against a solution of 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 0.01% (w/v) DM. Resin-bound r $\beta$ FXII, not subjected to refolding with the GSH/GSSG mixture, was eluted from the resin with a wash buffer containing 8 M urea and 1 M imidazole, and dialyzed against the same buffer as used for the other two forms of the protein. CD spectra of protein samples (0.3 mg/ml) in quartz cuvettes of 2 mm path length were recorded as an average of 5 scans with a Jasco J-720 spectropolarimeter over a range of 190–250 nm on a millidegree ellipticity scale. For comparison, they were transformed into the molar ellipticity [ $\theta$ ] scale using mean weight residue and protein concentration values.

### Measurement of amidolytic activity

Activities of r $\beta$ FXIIa and human  $\beta$ FXIIa were measured, as described previously [27]. Human  $\beta$ FXIIa or r $\beta$ FXIIa was added into a reaction buffer containing 0.1 M Tris-HCl, pH 8.0, 200  $\mu$ g BSA, and 100  $\mu$ M S2302 or S2222, the latter not being a substrate for kallikrein [2]. The total volume of the reaction was 1 ml. The catalytic activity was determined by monitoring the rate of hydrolysis of the chromogenic substrate at 405 nm. Protein concentrations were determined by the Pierce BCA protein assay. Relative activities of r $\beta$ FXIIa and human  $\beta$ FXIIa were calculated from  $\Delta A_{405}$  per s values, using the molecular weights of 32.6 kDa for r $\beta$ FXIIa and 28 kDa for human  $\beta$ FXIIa.

### Binding of rCMTI-V by r $\beta$ FXII and r $\beta$ FXIIa

rCMTI-V binding by refolded r $\beta$ FXII (zymogen) and r $\beta$ FXIIa (enzyme) was individually studied by isothermal titration calorimetry using a MicroCal OMEGA calorimeter. Refolded r $\beta$ FXII, before or after kallikrein treatment (activation), in a solution of 20 mM Tris-HCl, pH 8.0, 10 mM NaCl was placed in a 1.38 ml sample cell, the protein concentration being 0.013 mM. A 250  $\mu$ l syringe loaded with 0.465 mM rCMTI-V in the same buffer was used for a series of automatic injections of 10  $\mu$ l each into the protein solution. After each injection, a 250 s pause was allowed for reaching the baseline. Heat produced due to dilution was measured by injecting the rCMTI-V solution into the sample cell from which the protein was omitted. For each titration step, the heat of dilution was subtracted from the corresponding rCMTI-V binding data of the protein. Similar titrations were carried out with bovine trypsin. Data were fit to appropriate binding models and thermodynamic parameters were determined from nonlinear least-squares fits, using the ORIGIN software.

## Results

### PCR cloning of His(6)- $\beta$ FXII (r $\beta$ FXII)

The cDNA for the catalytic domain of human FXII (Asn<sup>335</sup> to Ser<sup>596</sup>), namely  $\beta$ FXII, was engineered by PCR in such a way as to contain two specific restriction sites (*Eco*RI/*Hind*III) for insertion into the *E. coli* expression vector pET28a(+). The steps involved in this procedure are summarized in Fig. 1. The vector has a 39 amino acid residue-upstream sequence to  $\beta$ FXIIa that includes an N-terminal His(6)-tag and a thrombin cleavage site to facilitate isolation and purification of the recombinant protein and to enable proteolytic removal of the His(6)-tag. The molecular weight of the whole recombinant protein was calculated to be 32.6 kDa as per the GCG program (University of Wisconsin).

### Distribution in bacterial cells and purification of expressed r $\beta$ FXII

Both the supernatant and pellet of the cell lysate were examined for the presence of the expressed recombinant protein. As shown in Fig. 2, almost all of the expressed recombinant protein was present in inclusion body of the pellet. The pellet was solubilized with a buffer containing 8 M urea and r $\beta$ FXII was isolated using Ni<sup>2+</sup>-chelated resin. This one-step purification yielded pure r $\beta$ FXII. The apparent molecular weight of the expressed recombinant protein was estimated to be about 35 kDa from the SDS-PAGE pattern (Fig. 2). The yield of r $\beta$ FXII ranged from 8 to 10 mg/liter of cell culture.

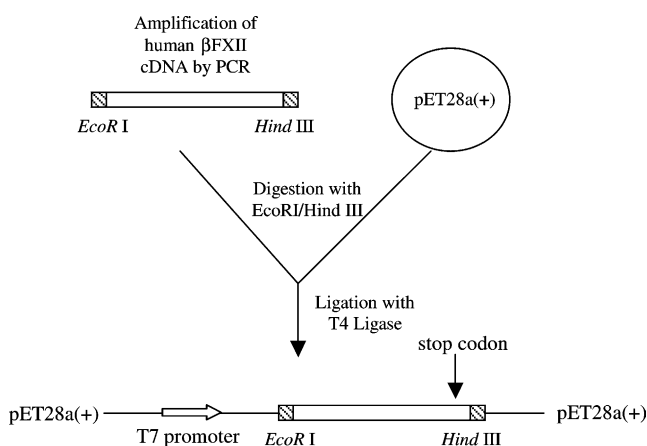


Fig. 1. Cloning strategy and construction of the expression vector for  $\beta$ FXII, the catalytic domain of human FXII. Vector pET28a(+)- $\beta$ FXIIa contains a T7 promoter, an N-polyhistidine tag, a thrombin restriction site, followed by a 795 base-pair coding sequence of human  $\beta$ FXII, and a stop codon.

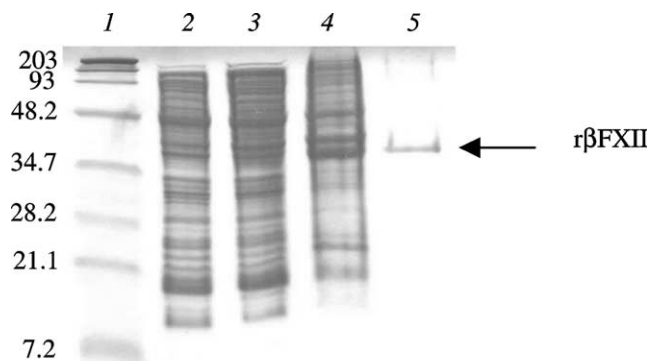


Fig. 2. SDS-PAGE of cell lysate and purified His(6)- $\beta$ FXII. Lane 1, pre-stained SDS-PAGE standards. Lane 2, *E. coli* cells containing pET-28a(+) vector without insert as control. Lane 3, supernatant of the cell lysate. Lane 4, pellet of the cell lysate. Lane 5, purified His(6)- $\beta$ FXII. Bio-Rad 16.5% Tris-tricine ready gel was used.

### CD spectra of express, refolded, and activated r $\beta$ FXII

Fig. 3 presents CD spectra of expressed r $\beta$ FXII, refolded r $\beta$ FXII, before and after kallikrein treatment, and human  $\beta$ FXIIa. The spectrum of the zymogen (refolded r $\beta$ FXII) is characterized by two minima at 208 and 220 nm, and a maximum at 192 nm which are diagnostic of the  $\alpha$ -helical structure [28,29]. These features become more obvious in the CD spectrum of r $\beta$ FXIIa, the enzyme form prepared by incubation with kallikrein. This CD spectrum, as expected, nicely matches that of active human  $\beta$ FXIIa. In sharp contrast, the CD spectrum of expressed r $\beta$ FXII, which has nine disulfide bridges and which was not treated with the GSH/GSSG system, appears to be not properly folded, if at all, and is characterized by a negative maximum at 198 nm, which is typical of denatured proteins [29].

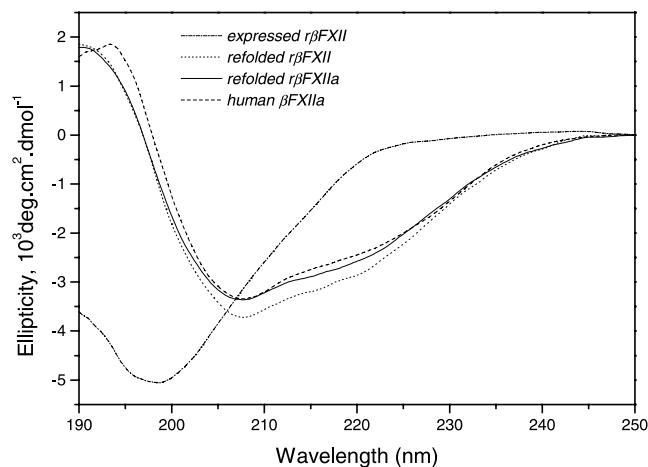


Fig. 3. CD spectra of refolded r $\beta$ FXII before (---) and after (—) activation by kallikrein. The CD spectrum of expressed r $\beta$ FXII (—) not subjected to GSH/GSSG treatment and human  $\beta$ FXIIa (· · ·) is also shown. The protein concentration was about 0.2 mg/ml in 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 0.01% (w/v) DM.

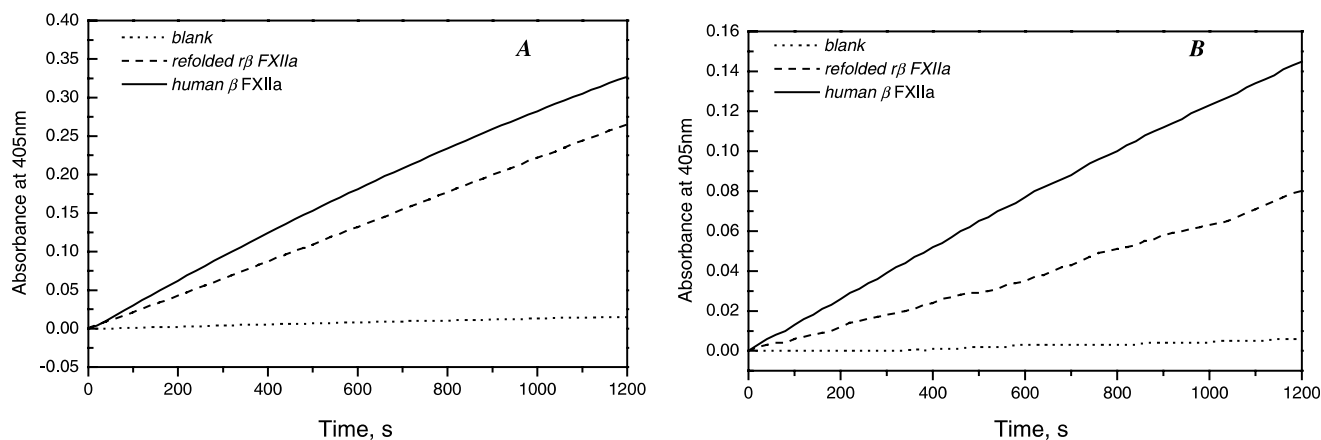


Fig. 4. rβFXIIa hydrolysis of substrates S2302 (A) and S2222 (B). The reaction buffer is 0.1 mM Tris-HCl, pH 8.0, 0.1 mM S2302 or S2222, 0.02% BSA, and 50  $\mu$ l sample at a concentration of 0.034 mg/ml (—). The same volume of the blank (see Materials and methods) used as control (···) and the same amount of human βFXIIa was used for comparison (—). Relative activities were calculated from  $\Delta A_{405}$  per s values, using a molecular weight of 32.6 kDa for rβFXIIa and 28 kDa for human βFXIIa: rβFXIIa exhibits ~91% activity toward S2302 and ~67% toward S2222.

#### Activity of refolded His(6)-βFXIIa

Refolded rβFXII did not hydrolyze the chromogenic substrate, S2302 or S2222 (data not shown), as expected of a zymogen. However, incubation of the recombinant zymogen with kallikrein, which activates FXII in vitro [1], results in the formation of the enzyme form, rβFXIIa, which catalyzes the hydrolysis of both S2302 and S2222, similar to human βFXIIa (Fig. 4). Relative to human βFXIIa, rβFXIIa is found to exhibit ~91% activity toward S2302 and ~67% activity toward S2222.

#### Binding of rCMTI-V by the zymogen and enzyme forms of rβFXII

rCMTI-V is a specific inhibitor of human βFXIIa [27]. To characterize the binding pocket in the inactive and active forms of rβFXII, isothermal calorimetric titration of rCMTI-V was carried out with rβFXII, before and after activation with kallikrein. The active form rβFXIIa binds rCMTI-V in a 1:1 molar ratio, as expected (Fig. 5). The binding thermodynamic parameters are collected in Table 1. ITC experiments with the zymogen, rβFXII, under similar conditions, did not yield any results indicative of binding, thus, suggesting a much weaker binding, if at all. Microcalorimetric titration of rCMTI-V with bovine trypsin (not shown) indicates a much stronger binding (Table 1). The binding process is facilitated by both enthalpy and entropy changes. The tighter binding of rCMTI-V by trypsin, as compared to rβFXIIa, results from entropy gain (Table 1).

#### Discussion

Bacterial expression systems provide a convenient and economical means of producing recombinant pro-

teins [30,31]. Earlier functional studies of deletion mutants of FXII employed a vaccinia virus expression system and the reported yield of the recombinant catalytic domain was of the order of submilligram/liter of cell culture [9], thus, being inadequate for biophysical investigations. However, many, if not all, recombinant proteins accumulate as insoluble, biologically inactive forms when overexpressed in bacterial cells [31–33]. Moreover, the expressed protein in inclusion body tends to aggregate during refolding [30,31], thus, hampering the refolding process.

Dominant interactions between exposed hydrophobic patches and formation of incorrect intramolecular disulfide links prevent a recombinant protein from attaining its native conformation. Immobilization of unfolded polypeptide chains on a matrix has been considered to be an efficient way to prevent inter-chain interactions and, hence, aggregation [30,31]. In the present work, removal of urea from the expressed protein by dilution alone did not result in the formation of correct disulfide bonds, as evidenced by the CD spectrum that is characteristic of a denatured protein (Fig. 3) and the absence of amidolytic activity in the kallikrein-treated recombinant protein. Expressed rβFXII that was purified and still bound to the His-Bind Resin with Sepharose as the matrix and iminodiacetic acid as the ligand arm was directly subjected to refolding with the GSH/GSSG mixture in the refolding buffer to serve as the “oxido shuffling” reagents [31,34]. This led to the formation of correct disulfide bonds on resin-supported rβFXII and resulted in the native folding of the recombinant protein, as indicated by the similarity of its CD spectrum to that of human βFXIIa (Fig. 3) and its ability to hydrolyze chromogenic substrates (Fig. 4) following digestion with the in vivo activator, kallikrein.

Evidence for the correct formation of the binding site in the kallikrein-treated rβFXII is provided by the 1:1

Table 1  
Thermodynamics of binding of rCMTI-V by r $\beta$ FXIIa and trypsin at 25 °C, pH 8.0

Protein	Number of binding sites	$K_d$ (M)	$\Delta G^0$ (kcal/mol)	$\Delta H^0$ (kcal/mol)	$T\Delta S^0$ (kcal/mol)
r $\beta$ FXIIa	$1.06 \pm 0.01$	$1.7 \times 10^{-6}$	-7.86	-4.59	+3.27
Trypsin	$0.96 \pm 0.01$	$4.9 \times 10^{-8}$	-9.97	-4.29	+5.68

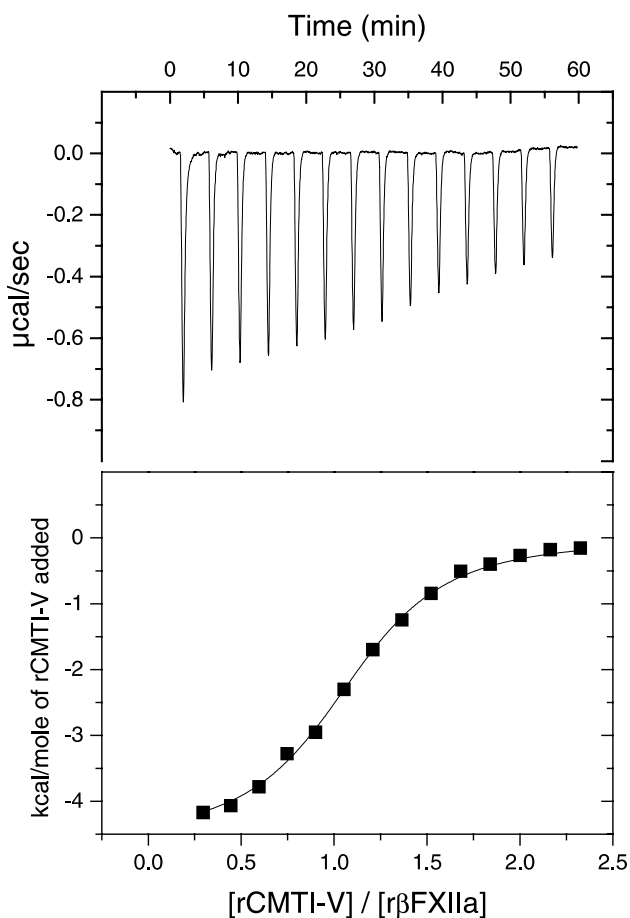


Fig. 5. Isothermal calorimetric titration of rCMTI-V with r $\beta$ FXIIa at 25 °C, pH 8.0; [r $\beta$ FXIIa] = 0.024 mM and [rCMTI-V] = 0.465 mM. A 250  $\mu$ l syringe loaded with rCMTI-V solution provided a series of automatic injections of 10  $\mu$ l each into the sample cell containing r $\beta$ FXIIa solution, and heat changes were recorded (upper panel). Corresponding heat changes were measured for injections of rCMTI-V into the sample cell containing only the buffer solution, but no r $\beta$ FXIIa (data not shown) and subtracted from the respective data obtained with the r $\beta$ FXIIa sample to determine heat changes due solely to rCMTI-V binding. These results are shown in the bottom panel. The line drawn through the data points represents the nonlinear least-squares fit and the computed thermodynamic parameters are given in Table 1.

stoichiometric binding of the specific inhibitor, rCMTI-V by r $\beta$ FXIIa, as monitored by microcalorimetric titration (Fig. 5), in accordance with the standard “lock and key” mechanism of serine protease inhibition [35]. Activation-triggered conformational changes occur at the catalytic site of the recombinant zymogen, as indicated by the CD spectra of r $\beta$ FXII and r $\beta$ FXIIa (Fig. 3)

and a much weaker binding of rCMTI-V by r $\beta$ FXII, as implied by microcalorimetry titration.

It is observed that compared to human  $\beta$ FXIIa, r $\beta$ FXIIa shows somewhat reduced amidolytic activity: 91% with S2302 and 67% with S2222 (Fig. 3). There are two factors that could account for this: (1) the recombinant protein has an additional carrier sequence of 39 amino acid residues at the N-terminal; (2) human  $\beta$ FXIIa has one glycosylated Asn [1], whereas r $\beta$ FXIIa is nonglycosylated. Interestingly, the enthalpy change of enzyme:rCMTI-V complex formation is almost the same for trypsin and r $\beta$ FXIIa, and the decreased binding affinity of  $\beta$ FXIIa, relative to trypsin, is entropy-related (Table 1). Active site-bound water molecules, and hence, the nature of binding pocket side-chains, are therefore anticipated to influence the binding affinity [36]. Instead of a Ser in the binding pocket of trypsin, an Ala occurs in FXII, thus, decreasing the hydrophilicity of the binding site [6]. The *E. coli* expression system reported here is thus expected to be quite useful for delineating structure–function relationships and basis of specificity and regulation of r $\beta$ FXIIa by the application of site-directed mutagenesis and biophysical techniques.

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