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Regulation of Trypsin Activity by Peptide Fraction of an Aqueous Extract of Human Placenta Used as Wound Healer

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An aqueous extract of human placenta, used as wound healer, shows stabilization of trypsin against autodigestion as one of the peptides of the extract binds very strongly with the protease. Trypsin retains 40% of activity at constant level between 20 and 26 days in presence of the extract against complete inactivation in its absence. Inhibition of esterolytic activity and inability to react with *p*-nitrophenyl-*p*'-guanidinobenzoate, HCl, an active site directed reagent, by trypsin in presence of a peptide fraction of the extract indicated blocking of the catalytic site of the enzyme. Rayleigh scattering, size-exclusion HPLC, fluorescence resonance energy transfer, and surface plasmon resonance show that fibronectin type III-like peptide present in the extract interacts with trypsin. The peptide–trypsin complex is dissociated in presence of high concentration of substrates. Thus, regulation of trypsin activity by the placental extract is evident.

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The human placenta produces a wide range of bioactive peptides and proteins that play essential roles in various cellular processes for fetal growth and development. Many of these placental proteins/peptides possess potent therapeutic properties; e.g., a glycoprotein is involved in corneal epithelial wound healing by serving as an extracellular matrix component (Yeh et al., 2005). A number of placental growth factors are endowed with healing potentials (Roberts et al., 1986; Brew et al., 1995; Lyall et al., 2001); such as placenta growth factor (PlGF) promotes neoangiogenesis associated with cutaneous wound repair (Failla et al., 2000). Placenta derived proteins and peptides are also used to alleviate oxidative stress related diseases, in curing chronic non-healing wounds of diabetic patients as well as in post-surgical dressings and high degree of burn injuries (Togashi et al., 2002; Cianfarani et al., 2006). Thus, various extracts of human placenta are used for curing several ailments and its precise therapeutic action depends on the method of preparation. However, only the aqueous extract shows potent wound healing activity (Tonello et al., 1996; Sur et al., 2003; Wu et al., 2003; Chakraborty et al., 2009).

The physiology of wound healing is a complicated process wherein different events operate in overlapping phases. These are inflammation (debridement), proliferation, and regeneration (epithelialization). Debridement comprises detachment and removal of necrotic tissue and foreign materials interfering with normal healing (Steed et al., 1996; Falanga, 2001). They are removed to create a platform for healthy tissue to grow. This phase is the foundation for the succeeding rebuilding steps to occur. During proliferation, a filament or template is constructed based on which tissue matrix forms. The matrix material is initially made up of fibrinogen and fibronectin (Witte and Abarbul, 1997; Diegelmann and Evans, 2004). Cell proliferation aimed at the generation of newly replaced tissues to fill and resurface the defect dominates this phase. Finally, regeneration occurs where readjustments take place to regain tensile strength of the newly formed healthy tissues (Gurtner et al., 2008).

Proteases play important roles in wound healing by regulating the balance between tissue degradation and regeneration (Lund et al., 1999; Moali and Hulmes, 2009). Slow removal of the necrotic tissue due to insufficient protease activity delays the onset of healing. On the other hand, an excess of protease during proliferation destroys growth factors and their receptors, as well as inhibit angiogenesis and breakdown granulation tissue resulting in tissue damage (Schultz and Mast, 1998; Trengove et al., 1999). A number of matrix-metallo proteases and their inhibitors control these intricate processes (Xue et al., 2006; Gill and Parks, 2008). Thus, regulation of proteolytic activity is critical for efficient scarless repair. Proteolytic enzymes like trypsin, fibrinolysin, collagenase, papain, and bromelain enhance healing of chronic wounds by accelerating the debridement phase and hence reduce inflammation (Carson et al., 2003; Hwang and Ivy, 2006; Ramundo and Gray, 2009).

It is our ongoing endeavor to characterize an aqueous extract of human placenta used as a licensed drug for wound healing in terms of its constituents and mechanisms of action (Chakraborty et al., 2009). Consistency of its manufacturing process (Datta and Bhattacharyya, 2004a), anti-microbial property against commonly occurring pathological organisms (Datta and Bhattacharyya, 2005), ability for in vitro NO induction in mouse peritoneal macrophages (Chakraborty et al., 2006), enhancement of cell adhesion (Nath and Bhattacharyya, 2007), and presence of functional NADPH (Datta and Bhattacharyya, 2004b; De et al., 2009) have been established. Further, a fibronectin type III (FN)-like peptide has

Abbreviations: FRET, fluorescence resonance energy transfer; SE-HPLC, size-exclusion high performance liquid chromatography, SPR, surface plasmon resonance; DN-PF, dansylated-peptide fraction.

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been isolated that contains the signatory RGD sequence required for cell adhesion (Chakraborty and Bhattacharyya, 2005; Nath and Bhattacharyya, 2007). These properties are directly or indirectly supportive to wound healing activity of the extract. Since the extract is a multi-component system and a number of pathways could trigger wound healing, its involvement might have different facets. Here, we demonstrate that a peptide fraction of the extract regulates the activity of trypsin, an important blood protease.

Materials and Methods

Placental extract

An aqueous extract of human placenta prepared from fresh term placentae using a single hot- and cold-water extraction followed by sterilization was used. Albert David Ltd., Calcutta, supplied the extract as sold under the trade name "Placentrex" (Datta and Bhattacharyya, 2004b). It contains 0.2% benzoyl alcohol as preservative that does not interfere with the experiments presented here. Pool of placental peptides was isolated as described in (De et al., 2009).

Reagents

Trypsin, azoalbumin, *N*-α-tosyl L-arginine methyl ester (TAME), *p*-nitrophenyl-*p*'-guanidinobenzoate (*p*-NPGB, HCl), *N*-bromosuccinimide (NBS), soyabean trypsin inhibitor (STI), bovine pancreatic trypsin inhibitor (BPTI), β-mercaptoethanol (β-ME), phenylmethyl sulphonyl fluoride (PMSF), 2,4,6-trinitrobenzenesulfonic acid (TNBS), dansyl chloride (DNS-Cl), 1,2-cyclohexanedione (CHD), yeast alcohol dehydrogenase, ovalbumin, carbonic anhydrase (bovine erythrocytes), myoglobin (horse heart), lysozyme (chicken egg white), human insulin, dialysis membrane (normal and benzoylated having cut off range 12 and 2 kDa, respectively) were obtained from Sigma (St. Louis, MO). EGTA and trichloroacetic acid (TCA) were from E-Merck, Darmstadt, Germany.

Protease assay

Trypsin activity was measured after incubating with 0.5% azoalbumin in 0.1 M Na-phosphate, pH 7.5 containing 0.001 M EDTA, and 0.01 M β-mercaptoethanol at 37°C for 1 h. Proteolysis was terminated by adding 10% TCA. An equal volume of 0.5 N NaOH was added to the supernatant after centrifugation and generated peptides were estimated at 440 nm (Plantner, 1991).

Esterase assay

Esterolysis of trypsin was followed using 1 mM of TAME in 40 mM Tris–HCl, pH 7.8 containing 10 mM $CaCl_2$ at 247 nm. Linearity of the hydrolysis reaction was maintained for 5 min (Hummel, 1959). Spontaneous hydrolysis of the reagent served as control.

Active site titration

p-NPGB can distinguish active and inactive forms of trypsin by selectively modifying the catalytic site serine residue (Kézdy and Kaiser, 1970). Quantification of the product was followed using $\varepsilon^{M}_{410\,nm} = 6{,}000$. The reagent (0.05 M) was dissolved in dimethylformamide and diluted with acetonitrile to make a working solution of 0.01 M. Reaction was initiated by the addition of 250 μM of *p*-NPGB containing trypsin (0.05 mg/ml) in 0.1 M Tris–HCl, pH 8.3 to a final volume of 1 ml, and followed for 300 sec at 25°C. Non-enzymatic hydrolysis of the reagent served as control. The increment of absorption of 0.30×10^{-5} M trypsin after *p*-NPGB modification corresponded to 0.25×10^{-5} M of the product. Thus, within limits, 83.3 ± 0.7% of the trypsin used was catalytically functional (n = 3).

Chemical modification

Dansylation. The isolated peptide fraction (2 mg/ml) was incubated with 0.1 mg/ml DNS-Cl in 0.1 M Na-carbonate, pH 9.0 at 37°C for 1 h (Gros and Labouesse, 1969). Excess unreacted reagent was removed by sephadex G-15 "spin column" equilibrated with 0.01 M Na-phosphate, pH 7.5 (Nath et al., 2003). Dansylation was confirmed from fluorescence (ex: 340 nm, em: 460 nm).

Modification of arginine. The peptide fraction was treated with 2 mM CHD in 0.2 M Na-borate, pH 9 at 37°C for 3 h (Davril et al., 1984). Excess reagent was removed by spin column.

Modification of lysine. The peptide fraction in 0.1 M Na-borate, pH 9.0 was treated with 0.1 mM TNBS at 37°C for 3 h (Habeeb, 1966; Pande et al., 1985). Modification was quantified using $\varepsilon^{M}_{367\,nm} = 11{,}000$ for the released trinitrophenyl group.

Modification of serine. Trypsin was treated with 0.5 mM PMSF for 1 h at 37°C in 0.05 M Na-phosphate, pH 7.5. Excess reagent was decomposed spontaneously by hydrolysis (Yoo et al., 2004).

Modification of tryptophan. Trypsin (2.5 mg/ml) in 0.1 M Na-phosphate, pH 7.5 was modified by drop wise addition of 0.25 ml of NBS from a stock of 0.32 mg/ml with vigorous stirring for 15 min at 4°C in the dark. Excess reagent was removed by dialysis (Hirasawa et al., 2005). For complete modification of tryptophan residues, BSA (50 mg/ml) was unfolded and reduced by 8 M urea containing 2 mM β-ME at 37°C for 16 h in the same buffer. Subsequently NBS modification was done as stated. Complete modification of tryptophan was ensured from fluorescence where no emission was observed (ex: 295 nm; em: 310-500 nm).

Rayleigh scattering

It was measured using a Hitachi F 3010 spectrofluorimeter (ex and em: 500 nm; slit widths 5/5 nm). For a single measurement, scattering was followed for 300 sec and the lowest intensity observed was noted (Brahma et al., 2009).

FRET analysis

FRET between trypsin (donor, ex: 295 nm; em: 300–450 nm) and DN-PF (acceptor, ex: 340 nm; em: 400–500 nm) was followed after exciting trypsin-DN-PF complex at 295 nm. FRET profiles were generated by gradual addition of DN-PF to trypsin (0.1 mg/ml) in 10 mM Na-phosphate, pH 7.5. No intramolecular FRET between tryptophan and dansyl residues of the peptide was observed.

Size-exclusion (SE)-HPLC (Hummel–Dreyer method)

In this method, binding between two proteins of significant mass difference could be detected by SE-HPLC where the equilibrating buffer contains one of the interacting components. When the second component is applied, a peak appears corresponding to the combined mass of the adduct along with a dip or trough corresponding to the mass of the depleted component of the buffer (Berger and Girault, 2003; Soltés, 2003). A Protein Pak 125 SE-column (Waters, 13 × 78 mm, fractionation range 5–80 kDa) was used to follow the interaction between trypsin and the peptide fraction. The column was equilibrated with 50 mM Na-phosphate; pH 7.0 containing 0.1 mg/ml of trypsin at a flow rate of 0.8 ml/min and elution was followed at 220 nm. The column was pre-calibrated with reference proteins such as lysozyme, myoglobin, carbonic anhydrase, trypsin, ovalbumin, BSA, and yeast alcohol dehydrogenase wherein a linear dependency was obtained between log M_w and V_t ($R^2 = 0.922$).

Surface plasmon resonance (SPR) analyses

SPR was performed using a Biacore 3000 instrument (GE Healthcare, Uppsala, Sweden) (Biacore Tutorial, 2008). Trypsin (6.72×10^{-10} M) as ligand was covalently immobilized to the surface of a CM5 sensor chip using the amine-coupling protocol. The analytes used, their concentrations and flow rates at which injected over flow cells were as follows: placental peptide fraction, 8.75×10^{-7} M and fibronectin IIIc, 3.47×10^{-9} M at 5 μl/min; and STI, 5.2×10^{-10} M at 2 μl/min. In each case, the chip was

regenerated using 0.3–1.0 M NaCl depending upon the strength of binding between trypsin and the analyte. Binding response was observed by adding increasing concentrations of placental peptide fraction ($4.86–24.31 \times 10^{-7}$ M) over bound trypsin (6.72×10^{-10} M). Data analyses were performed using BiaEvaluation 4.1 software. Non-specific binding between the analyte and the sensor chip was negligible.

Other methods

Peptide concentration was determined based on absorption at 225 nm with reference to standard proteins (De et al., 2009). Stock solution of lyophilized trypsin (5 mg/ml) was prepared in HCl, pH 3.0 and was preserved at 4°C for a day's work where it was reversibly inactivated. Exact concentration was determined from $\varepsilon^{1\%}_{280nm} = 12.9$ (acidic pH). "Trypsinized placental extract" was prepared by treating the supplied extract with trypsin (1:25, wt/wt) for 24 h at 37°C and at pH 8.8. To avoid interference of subsequent trypsin treatment, no inhibitor was applied. A Hitachi spectrofluorimeters model F-4500 was used for fluorescence measurements. Optical absorbances were recorded with a Specord 200 spectrophotometer (Analytic Jena, Germany).

Results

Trypsin activity in placental extract

Short term incubation. Apart from natural inactivation, proteases are degraded by autodigestion. Trypsin (0.5 mg/ml) was incubated in Tris–HCl, pH 8.8 for 1 h at 37°C in presence of the supplied placental extract. Considering the activity of trypsin at 0 h as 100%, residual activity of the enzyme in presence and absence of the extract were 95 ± 3 and 60 ± 5%, respectively (n = 3). This observation leads to two possibilities. First, placental extract may contain a trypsin activation factor, the possibility of which is rare. Second, the extract could induce stabilization of trypsin. In case of autodigestion-prone proteolytic enzymes, prevention of autodigestion is an important consideration for residual activity as compared to stabilization of structures. Thus, the extract may prevent autodigestion or stabilize the functional site of trypsin against thermal denaturation or turnover related inactivation.

Autodigestion of a protease enters into kinetic competition and hence reduced if the enzyme has access to its substrates. Since the proteins/peptides of the placental extract may serve as substrates for trypsin, the extract was exhaustively digested with trypsin so that all trypsin sensitive bonds were hydrolyzed leaving no scope for further interaction with the enzyme. The excess trypsin was destroyed by heating. When "placental extract" was replaced by the "trypsinized placental extract," stabilization of trypsin was observed.

Ca^{+2} ions play an important role in trypsin stabilization (Kotormán et al., 2003; Bruniusa and Sundbom, 2004). Thus, it was necessary to determine if the extract supplemented the required ion. Incubation of the extract with EGTA as chelator of Ca^{+2} had the same effect on trypsin stabilization negating the role of Ca^{+2}. To elucidate the size of the stabilizer, if any, the extract was dialyzed using normal and benzoylated dialysis membranes before incubation with trypsin. Residual activity of trypsin was reduced to zero in the later case. Thus, components of 2–12 kDa appear to interact with trypsin.

Long-term incubation. Trypsin (0.1 mg/ml) was incubated at pH 8.8, 37°C for 26 days in presence and absence of the extract and the time course of inactivation was followed (Fig. 1). It showed that the activity was reduced to 1–2% by 20 days in absence of the extract, otherwise; it was reduced to 45 ± 5% after 14 days and remained constant for the next 12 days (n = 3, with different batches). This observation is significant because it indicated that some factor/s of the extract reversibly protected trypsin against autodigestion. Stabilization of trypsin from structural aspect was not apparent. Had it been so, the native structure of trypsin would be autodigested even faster.

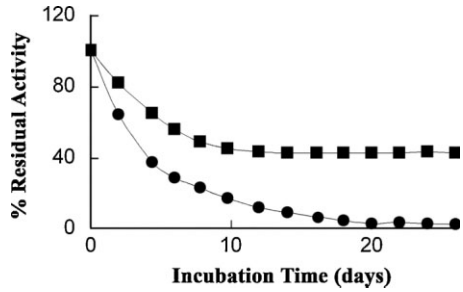

Fig. 1. Time course of inactivation of trypsin (0.1 mg/ml) in presence (■) and absence (•) of placental extract at 37°C for 26 days at pH 8.8. Residual activity was measured from azoalbumin assay. The experiment was performed three times, with different batches. Variation was 45 ± 5%.

Esterolysis. Stabilization of trypsin against autodigestion is possible if the stabilizing component binds at the autodigestion initiation site marked by a lys- or an arg-residue or at the catalytic site. This was distinguished by TAME, an ester of M_w 378.87 Da. Results indicated that with 0–5-fold concentrated placental extract, inhibition of esterolysis was markedly increased up to 85 ± 6% compared to the control (n = 4) (Fig. 2A). Thus, binding at catalytic site by a component of placental extract was indicative. These observations indicated the possible involvement of stabilizing component/s of the extract.

Regulation of trypsin activity by peptide fractions

Several lines of evidence suggested that the placental peptide fraction caused stabilization of trypsin. First, peptides of <10 kDa are the major constituents of the extract (Chakraborty and Bhattacharyya, 2005) that are natural substrates/inhibitors of trypsin; second, this size corresponds to the dialysis experiment; and third, background evidence supports that peptides of similar size inhibit and stabilize proteases (Ingallinella et al., 1998; Munekiyo and Mackessy, 2004). Trypsin was incubated with placental peptide fraction at 1:1 and 2:1 mol/mol ratio and was allowed to autodigest at pH 8.8, 37°C. After 48 h, residual activities were 45 ± 5 and 80 ± 5%, respectively (n = 3; with different batches). Under identical conditions, only 20 ± 4% of activity was retained in absence of the peptide (Fig. 2B). The results indicate that trypsin activity was favorably retained with increase in peptide concentration. An SDS–PAGE pattern of the digest also supports this. Higher concentration of trypsin in the incubate (0.5 mg/ml) in absence of the extract led to rapid disappearance of the protease band due to concentration dependent autodigestion (Fig. 2C).

Esterolysis of trypsin in presence of the peptide fraction also showed concentration dependent inhibition. By preventing small molecules like esters to act, the peptide seems to block the active site of trypsin.

Evidence of trypsin–peptide fraction interaction

Active site titration. To distinguish whether the peptide fraction interacts exclusively at the catalytic site of trypsin, specificity of *p*-NPGB was employed. The time course of a set of four reactions was followed; *p*-NPGB with buffer in presence

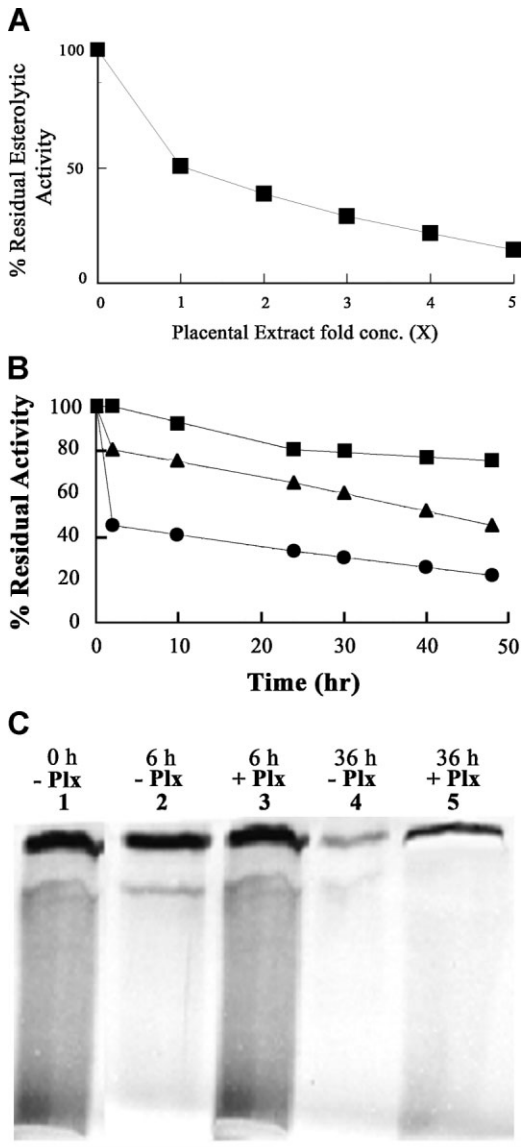

Fig. 2. (A) Inhibition of esterolytic activity of trypsin by 0–5-fold concentrated placental extract. Variation was 85 ± 6% (n = 4). (B) Stabilization of trypsin activity with trypsin: peptide fraction (mol/mol) ratios of 2:1(▲) and 1:1(■). An incubate in absence of the extract served as control (•). The experiment was repeated three times, with individual data point variation = ±5%. (C) 20% SDS–PAGE of 30 μg trypsin. Lanes 1–5 can be identified. (−) indicates in absence and (+) in presence of the extract. The time duration of the incubates have been indicated above in hour (n = 3). Trypsin concentrations, pH, and temperature of the incubates were 0.5 mg/ml, 8.8, and 37°C, respectively.

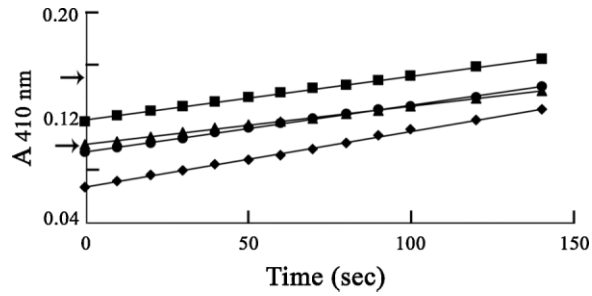

Fig. 3. Active site titration of trypsin. The time course of reactions are; non-enzymatic hydrolysis of *p*-NPGB (◆), *p*-NPGB with trypsin (■), *p*-NPGB with peptide fraction (•) and finally trypsin preincubated with peptide fraction followed by addition of *p*-NPGB (▲). Peptide fraction shows low absorption at 410 nm but it did not react with *p*-NPGB. The reaction condition has been mentioned in the text. The upper arrow indicates absorption of trypsin–placental peptide incubate in case of modification by the reagent, whereas the lower arrow indicates the actual absorption. This is a typical representation of a single set of experiment (n = 5). Deviation of individual data points was ±4%.

and absence of the peptide fraction as control; and *p*-NPGB with trypsin preincubated with and without the peptide fraction as test sample. In all sets, an immediate burst of absorbance within the dead time of mixing with *p*-NPGB was observed, clearly indicating that the reaction completed within this time period. Thereafter, spontaneous hydrolysis of the reagent was evident. However, the reaction of trypsin–peptide fraction complex was completely inhibited (upper versus lower arrow, Fig. 3). This indicated that in trypsin–peptide fraction complex, the catalytic site of trypsin is blocked.

Rayleigh's scattering. In dilute and non-interacting systems, scattering from proteins are additive. Any deviation, usually an enhancement, indicates interaction between proteins (Brahma et al., 2009). It was observed that when to a fixed concentration of peptide fraction (0.36 mg/ml) varying concentrations of trypsin (0.02–0.40 mg/ml) was added, an enhancement of scattering over the additive values was observed suggesting binding between the components (Fig. 4A).

FRET analysis. Strong FRET was observed when increasing concentration of DN-PF was added to trypsin (0.1 mg/ml). Gradual enhancement of emission intensity at 460 nm at the cost of quenching at 350 nm with the generation of a distinct isobestic point at ~387 nm was observed (upward, downward, and diagonal arrows, Fig. 4B). This indicated that the energy transfer was intra molecular. Since peptides have the general property of self-aggregation and association with proteins, interactions between DN-PF and three unrelated proteins like BSA, lysozyme, and ovalbumin was followed, where no FRET was observed. Thus, the interaction of DN-PF with trypsin appeared to be specific.

Further, donor–acceptor interaction sites have been identified using group specific reagents to modify serine and tryptophan residues of trypsin in one hand; and lysine and arginine residues of DN-PF on the other (Table 1). Results indicated that while the tryptophan residues of trypsin act as donor, arginine modified peptide does not accept energy from trypsin resulting in FRET.

SE-HPLC analysis. The isolated placental peptides being heterogeneous, demonstration of its interaction with trypsin using conventional SE-HPLC was difficult. As an alternate, Hummel–Dreyer method was applied where the column was equilibrated with trypsin (Berger and Girault, 2003). As a positive control, STI was applied and the chromatogram showed a peak corresponding to trypsin–STI conjugate (23.3 + 22.0 = 45.3 kDa) and a trough at $R_t = 14.51 \pm 0.5$ min where trypsin appeared (n = 3) (Fig. 5 Upper Part). When the peptide fraction was applied, a deep trough similar to the earlier position ($R_t = 14.66 \pm 0.18$ min; n = 6) appeared (Fig. 5 Lower Part). The depth of the troughs was proportional to the amount of peptides applied (result not shown). Since the elution pattern of the peptides was broad because of variable sizes and partial aggregated character, no single peak corresponding to trypsin–peptide conjugate could be identified.

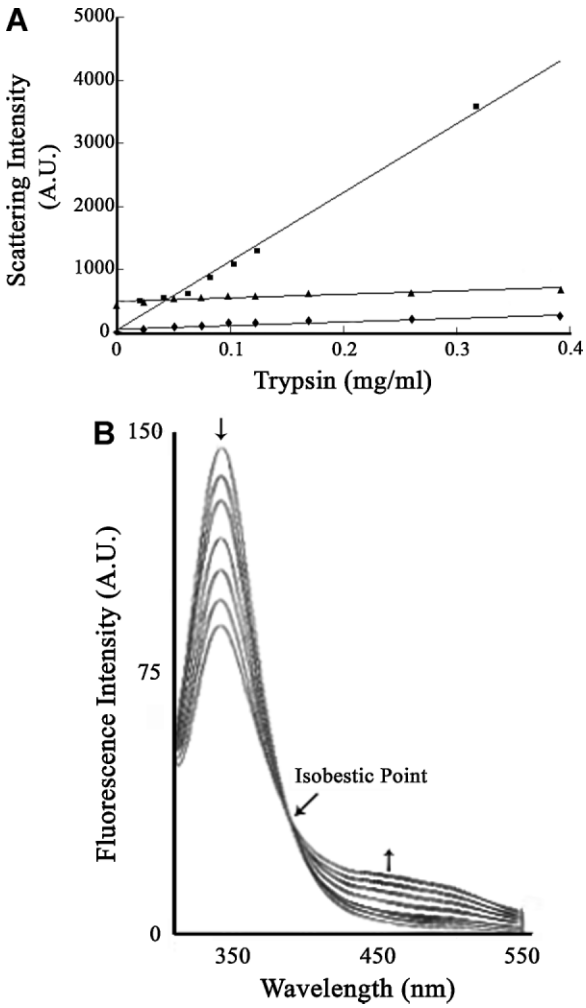

Fig. 4. (A) Tryspin–peptide fraction (PF) interaction as monitored by Rayleigh scattering. Increase in scattering intensity upon addition of trypsin to PF is indicated by (■). Scattering intensity of trypsin in absence of PF and theoretical additive scattering intensity in absence of interaction between PF and trypsin have been indicated by (◆) and (▲), respectively. Uncertainty of individual data points is ±3%. The whole experiment was repeated thrice, where identical results were obtained. (B) FRET between trypsin and dansylated peptide fraction. The downward arrow indicates quenching of fluorescence of trypsin at 350 nm while upward indicates an increase in the fluorescence intensity from dansyl group of peptide fraction at 460 nm. The diagonal arrow indicates the isobestic point at 387.0 nm. Variation of isobestic point was ±3 nm (n = 6).

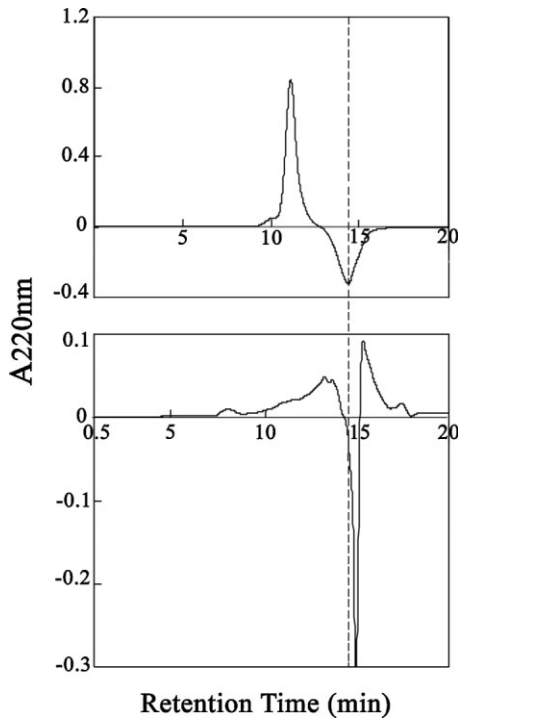

Fig. 5. Size-exclusion HPLC (Hummel–Dreyer). (Upper Part) Trypsin–STI interaction. The adduct of trypsin–STI conjugate (23.3 + 22.0 kDa) showed a peak at 11.6 min, while the trough appeared at 14.5 min corresponding to the retention time of trypsin. (Lower Part) Trypsin and 2× dilute peptide fraction showed a trough at 14.8 min. Peptide fraction being heterogeneous shows an unresolved profile on normal SE-HPLC and thus the trypsin-PF adduct could not be detected by this method. $R_t = 14.66 \pm 0.18$ min; n = 6. The dotted line across the two parts correspond to the elution position of trypsin. The whole experiment was repeated four times.

SPR analyses. Equilibrium dissociation constant and hence binding affinity between trypsin and its substrates were determined from the sensograms generated in SPR analysis. Since the extract contains fibronectin type IIIc as one of the peptides that bears sequence homology with STI or BPTI (described later), its interaction with trypsin was followed separately. Results showed that in case of STI, after dissociation of unbound ligands up to 450 sec under the experimental conditions, the response remained constant at 273 RU until 700 sec (Fig. 6 Upper Part). This yielded K_d of 1.75×10^{-9} M as compared to reported value of 2×10^{-10} M (Chen and Jang, 2004). Similar experiments with standard fibronectin type IIIc showed abrupt association followed by relatively rapid dissociation initially for 300 sec yielding K_d of 2.25×10^{-8} M. The remaining RU reflected the bound peptide (Fig. 6 Upper Part).

When peptide fraction was passed through the flow cell at increasing concentrations, in all the cases it was observed that after abrupt association, the dissociation profile showed an initial sharp drop in RU, but a fraction remained bound to trypsin as reflected from RU values after 700 sec, which thereafter remained fairly constant. Derived K_d was 2.84×10^{-7} M. Results indicated an increase in the amount of analyte bound to the ligand with increasing concentrations of the peptide fraction (Fig. 6 Lower Part; Lower Inset). After a certain concentration of the ligand applied, RU change was insignificant. This indicated the saturation point, beyond which no binding takes place (result not shown). The concentration dependence of peptide fraction was further reflected when analyte concentration (14–56 μg) was plotted against bulk refractive index. A linear dependence with regression

TABLE 1. FRET analysis of modified trypsin and placental peptide fraction

Modification reagent	Trypsin	Dansylated-PF	FRET (%)[1]
PMSF	+	−	98.0
Cyclohexanedione	−	+	3.0
TNBS	−	+	98.0
NBS	+	−	0.0
STI/BPTI	+	−	97.0

(+) indicates modified and (−) indicates unmodified.
[1]FRET 100% corresponds to trypsin–dansylated peptide fraction.

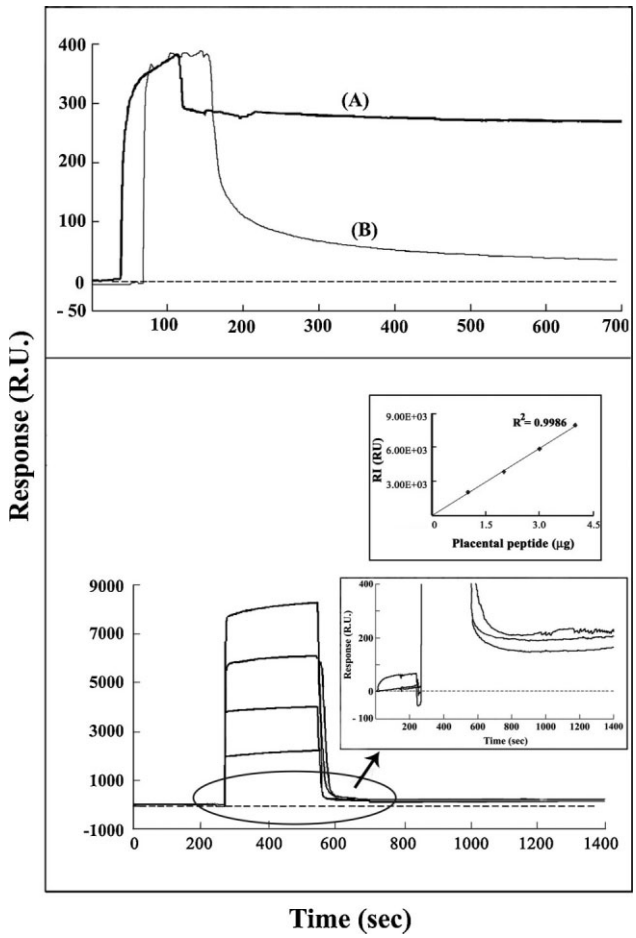

Fig. 6. Surface plasmon resonance analysis. (Upper Part) Sensogram response of STI (A, dark line) and fibronectin type IIIc (B, lightline) binding to trypsin. STI showed a stronger binding (273 RU) as compared to fibronectin (43 RU). (Lower Part) Senogram representing response generated with increasing concentrations of peptide fraction (14–56 μg) over a CM5 chip previously coated with trypsin. Upper Inset; Bulk refractive index (RU) versus concentration of peptide fraction. Linear dependence was obtained with $R^2 = 0.998$. Lower Inset; magnified portion (marked eclipse on Lower Part) of concentration dependence sensogram of peptide fraction on trypsin. The experiment was repeated thrice.

coefficient, R, of 0.998 was obtained (Fig. 6 Lower Part; Upper Inset).

Stability of trypsin–peptide fraction complex. To a solution of dansylated peptide fraction previously incubated with trypsin, an increasing concentration of STI was added. STI could not replace the peptide fraction bound to trypsin as evident from unaltered FRET profile. Alternatively, trypsin preincubated with STI could be replaced by increasing concentrations of DN-PF as evidenced from FRET. This indicated that the K_d of the peptide fraction is higher than or almost equal to that of STI for trypsin. Similar experiments with BPTI, where the binding with trypsin is even stronger, did not show significant dissociation of the peptide complex.

Since this type of near-irreversible binding might lead to complete inactivation of trypsin, the situation was evaluated in presence of high concentration of natural substrates like BSA. The stability of trypsin-DN-PF conjugate were largely monitored by FRET in presence of high concentrations of tryptophan depleted BSA to remove any background

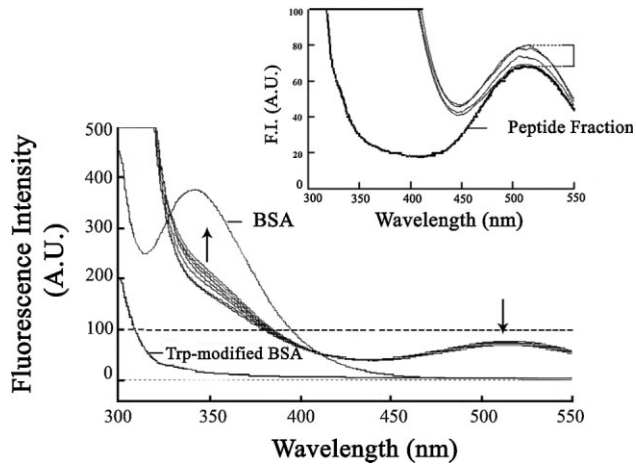

Fig. 7. Reversal of FRET by addition of excess substrate, i.e., tryptophan-modified BSA. Quenching of fluorescence emission of dansylated peptide fraction (DN-PF) after addition of modified BSA to the saturated complex of trypsin-DN-PF is indicated by the downward arrow at 520 nm. Concomitant increase of emission from trypsin is indicated by the upward arrow at 350 nm. Emissions of unmodified BSA as well as tryptophan-modified BSA have been indicated that shows non-interference of fluorescence emission by the substrate in this experiment. The dotted line between 0 and 100 indicates the portion that has been magnified in the inset. (Inset) Magnified portion of the FRET region. Quenching of fluorescence corresponding to the bracket indicates ~100% release of the interacting FRET partners. Emission from DN-PF as background has been indicated. 95 ± 3% reduction of FRET was observed (n = 5).

interferences. Results indicated that with increasing concentration of NBS modified BSA (5–8 mg/ml) to the complex, FRET intensity ($em_{max} = 520$ nm) of trypsin-DN-PF decreased gradually. Approximately 95 ± 3% reduction in FRET has been found as the BSA concentration reached at 7.8 mg/ml (Fig. 7 Inset).

Discussion

Intravenous trypsin can relieve the symptoms of many different inflammatory conditions, e.g., burns, rheumatoid arthritis, ulcerative colitis, atypical viral pneumonia, etc. Intramuscular enzyme injections were found to be beneficial in counteracting post-surgical swelling (edema), treating thrombophlebitis and lower back strain, and rapidly healing bruises caused by sports injuries (Deitrick, 1965). Trypsin may not act as an anti-inflammatory agent but as accelerants of the inflammatory process, thereby shortening its duration (Craig, 1975). It is documented that enterically-coated enzymes such as trypsin, chymotrypsin, or bromelain collectively called as debridase are orally active and have been used successfully for reducing inflammatory conditions (Rathgeber, 1971).

Considering the importance of proteases in wound healing and evidences of its use as an oral supplement, it was logical to ask if the placental extract has any influence on trypsin activity in vitro. This study reports fairly convincingly using both biochemical and biophysical tools that peptide fraction present in placental extract reversibly inactivates trypsin, preventing its autodigestion and thus stabilizing its activity (Figs. 1–7). Various systemic inhibitors regulate the activity of proteases during wound healing. It is already reported that many naturally occurring miniproteins act as inhibitors (Kolmar, 2009; Sommerhoff et al., 2010). It is known that the common trypsin inhibitor/s such as STI almost irreversibly inactivates trypsin. Preliminary findings indicated the role of the isolated peptide

fraction in stabilizing the activity of trypsin by interacting at its catalytic site. This association is quite strong as the K_d of trypsin–dansylated peptide (1:1 stoichiometric complex) could not be estimated using equilibrium dialysis experiment. Further, the trypsin–peptide complex could not be dissociated by STI or BPTI. Probably the most intriguing question of this study is that, if a peptide fraction binds so efficiently with trypsin that it cannot be dissociated by STI or BPTI, then how azoalbumin could replace the bound peptide with restoration of proteolytic activity in vitro? It has been demonstrated using tryptophan modified BSA that this substrate can indeed dissociate the trypsin–placental peptide complex to an extent of $95 \pm 3\%$ (Fig. 7).

In a different context, it has been reported that human fibronectin type III peptide has high efficacy of stimulating cell migration and wound repair (Cheng et al., 1988; Nath and Bhattacharyya, 2007). Since fibronectin type III-like peptide has already been identified as the major constituent of the extract (Chakraborty and Bhattacharyya, 2005) and it has sequence homology with trypsin inhibitor like BPTI (described later), SPR had been employed to study trypsin FN peptide interaction. SPR studies indicated the reversible nature of the binding phenomena exhibited by the isolated peptide fraction, which was also demonstrated in the control fibronectin type III peptide. This binding is not as strong as STI and the dissociation profile is relatively prolonged. Using placental peptide fraction at increasing concentrations, a linear response has been generated. Further, the K_d values of STI binding was much lower than that of fibronectin type III like peptide. This indicated that possibly the binding affinity is much higher as compared to normal substrates of trypsin yet it is reversible with K_d values comparable to those of standard substrates.

Finally, the electrostatic potential surface of trypsin, BPTI, and fibronectin type IIIc models were generated using MODELYN software (Fig. 8) (Majumder et al., 2006). Sequence alignment of fibronectin type III peptide and BPTI revealed considerable sequence homology between the two. Based on this finding, a model of fibronectin type III peptide had been generated and this was compared to that of BPTI. It had been observed that the region of BPTI that fit into the acidic groove on trypsin (marked in red) was similar to the binding site of the derived model of the fibronectin peptide. Analysis of the fibronectin model revealed basic regions (blue) similar to BPTI that apparently fit into the groove of the trypsin. It had further been observed that the binding site of the peptide is less basic as compared to that of BPTI; this possibly explains the comparatively weak binding by the fibronectin peptide, and hence the probable cause of reversible nature of the binding.

Further, sequence alignment of the known binding site (underlined in red; Fig. 8) on BPTI that might have homology with the peptide had been obtained by clustalW. The binding sequence of BPTI showed maximum score (33) to an arginine (R) rich region (27–32) of fibronectin peptide. The other arginine residues on fibronectin are at positions 5, 76, and 92. The R5 is flanked to the left by proline, which cannot be a favorable binding site for trypsin owing to steric hindrance. R92 is likely unfavorable owing to the lack of proper sub-site interaction required for trypsin binding. The other arginine residue in the fibronectin sequence is the RGD motif that is a known signature sequence for promoting cell adhesion. However, the query on clustalW always indicated a better fit with the R29–R32 stretch. Thus, it is indicative that the binding region of FN peptide with trypsin is the marked arginine region. Moreover, arginine being basic in nature, the binding sequence of fibronectin peptide which matches with the basic trypsin binding region of BPTI, further complies with previous modeling experiments. FRET experiments between FN peptide and trypsin also indicated involvement of an arginine in the binding (Table 1).

From these findings, we conclude that one or more peptides from human placental extract including fibronectin type III stabilize trypsin activity after strong association. In presence of excess substrate, the conjugate is dissociated. Thus, stabilization of trypsin activity is evident. The present observation cannot be extrapolated to in vivo situation, but protease regulation as required in wound healing is reflected.

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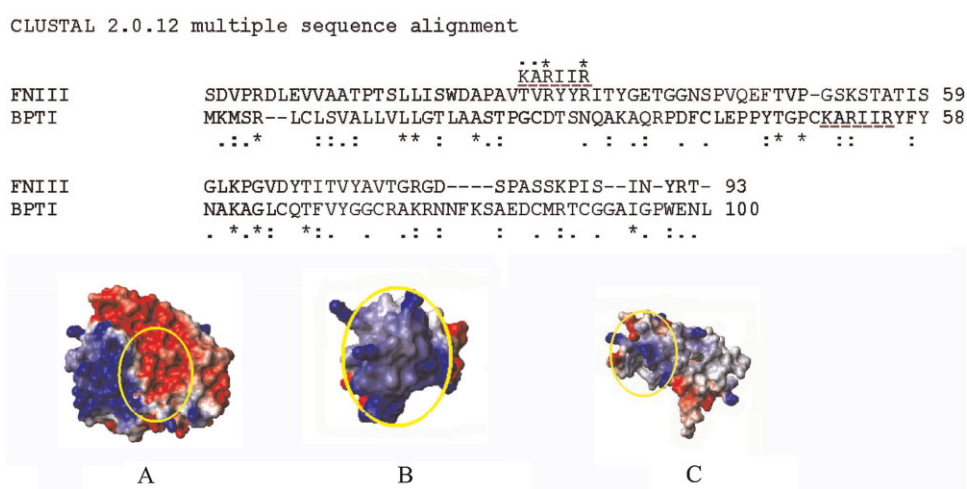

Fig. 8. Homology modeling of fibronectin type IIIc peptide (FN-peptide) as compared to BPTI with reference to trypsin: (A) Trypsin, (B) BPTI, and (C) fibronectin type IIIc peptide. Negatively and positively charged residues have been presented as red and blue, respectively, while white represents both hydrophobic and neutral polar residues. The binding site between trypsin and BPTI or the peptide has been marked by yellow circle. The sequence alignment between FN peptide (FN III) and BPTI derived from clustalW has been represented on the top. The "*" indicates identical residues, ":" and "." indicate highly similar and similar residues, respectively. The red dotted line under BPTI sequence represents its trypsin binding site. This sequence has maximum homology to FN III sequence 27–32, which is indicated on the top.

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