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Functional Mimicry of Protein A of *Staphylococcus aureus* by a Proteolytically Cleaved Fragment[1]

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Protein A (PA) of *Staphylococcus aureus* has an array of biological functions, such as antitumor, antitoxic, anticarcinogenic, immunomodulatory, antifungal, and antiparasitic properties. We have already established that a theoretical trypsin-digested peptide fragment of protein A (20-mer) mimics immunomodulatory and IgG binding property of PA. In the present report we have concentrated on a 16-mer chymotryptic fragment of protein A, which has a sequence of 13 amino acids in common with the previously studied 20-mer peptide. Molecular modeling study qualitatively predicted that both 20-mer and 16-mer peptides retain Fc binding ability from an interaction energy point of view. In the present study our aim was to understand whether this theoretically predicted 16-mer chymotryptic fragment could be formed in a real experiment and also to understand its biological activities. Chymotrypsin cleavage of PA at 37°C for 24 h produced four major fragments on reverse-phase HPLC. The amino acid analyses of each fragment show the absence of cysteine residue from all fragments, which justifies the absence of cysteine in PA. We also observed high content of aspartic acid and glutamic acid residues in all fragments. On gel-filtration chromatography the chymotrypsin cleavage of PA shows five peaks, one of which overlaps with our theoretically selected 16-mer peptide on superimposition. We verified the IgG binding capacity of 16-mer peptide by capillary electrophoresis. The 16-mer peptide also induces the production of TNFα and IL-1α in serum of mice. The above observations suggest that the 16-mer peptide may be produced by chymotrypsin cleavage and also that this peptide possesses some of the major biological properties of PA, such as IgG binding, TNFα and IL-1α elicitation, etc.

Key Words: **protein A; chymotrypsin; peptides; Fc binding; cytokines.**

Protein A (molecular weight 42 kD, 395 residues), a cell wall protein from *Staphylococcus aureus* precipitates IgG from several species via interaction with the Fc portion of Immunoglobulin G (IgG). It is used as a tool in the laboratory for isolation and purification of antibody molecules (1). This unique Fc binding property of PA was also utilized to remove the "blocking factors" from human cancer patients (2). Alternative PA binding sites on IgG have also been reported which were located to the Fab region of IgG (3). The Fab structures, unlike Fc, involved in PA binding are much less studied.

Diverse array of biological functions of PA were demonstrated, such as antitumor (4, 5), antitoxic (6), anticarcinogenic (7), Immunomodulatory (8), antifungal (9) and antiparasitic effects (10). Protein A has been demonstrated to act as a B and T cell mitogen (11, 12). It also induces production of different cytokines (8), which are well known biological modulators regulating cellular growth and differentiation on one hand, and apoptosis (13, 14) on the other. Moreover, PA can activate phase I and phase II biotransformation and detoxification enzymes (15, 16). During these studies (17) we have consistently observed that PA treatment leads to an increase in both peripheral blood and splenic lymphocyte population. It was also observed that PA expands CD4+/CD8+/CD19+/CD34+ cells in mice (18). PA induced activation of cell cycle shifting vis a vis proliferation in non-Hodgkin's lymphoma has also been reported (19).

The amino acid sequence of PA is comprised of five homologous IgG binding domains (from the N-terminus: E, D, A, B, and C respectively) followed by the C-terminal cell wall binding region X, which differs to a great extent from the other IgG binding active regions (20). A comparison of sequence data of different Fc-binding regions indicates not only a mutual homology but also internal homologies within the region (20). The B-domain of protein A, a 56-residue domain, binds the Fc portion of IgGs with dissociation constant (kD) of about 10–50 nM (1). X-ray (21) and NMR (22) studies showed that binding contacts are presented from

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helix 1 (Gln 9-Leu 17) and helix 2 (Glu25-Asp36) of the B-domain (PA). One of the reasons for the interest in PA–IgG interaction is that it may help to explain the structural basis of the certain biological activities associated with PA.

It was shown that some synthetic peptides containing sequences encompassed by staphylococcal PA could induce immunomodulation and cytotoxicity (23). The smaller functional versions would become synthetically accessible. Our hypothesis is that PA, being a foreign protein, may be subjected to proteolytic cleavage *in vivo*. It is thus possible that such degraded products might be functionally operative *in vivo*. Molecular modeling studies reported earlier (24) qualitatively showed that both 20-mer and 16-mer peptides may retain Fc binding ability from interaction energy point of view. Further the 20-mer peptide was shown to mimic many of the biological properties of protein A (25) In the present study, we have selected the 16-mer peptide derived from chymotrypsin digestion (theoretical) of B-domain among all five IgG binding homologous domains of protein A. This 16-mer peptide has a sequence of 13 amino acids in common with the previously studied 20-mer peptide. Our aim is to verify whether 16-mer peptide fragment is formed in reality by chymotrypsin cleavage and also to study biological properties, if any, associated with this peptide.

MATERIALS AND METHODS

Theoretical methods. "Peptide Map" and "Peptide Sort" programs available in Wisconsin sequence analysis package (Genetic Computer Group, ver. 8) were used to generate the peptide fragments following chymotrypsin digestion of protein A sequence (20).

Reagents. Freeze-dried protein A (5 mg/ml) was procured from Pharmacia fine chemicals, Sweden. Chymotrypsin, trifluoroacetic acid, acetonitrile and HPLC grade Water were obtained from E-Merk, Germany. Phenylisothiocyanate (PITC), standard amino acids and Human IgG were obtained from Sigma chemicals, U.S.A. 16 amino acid (16-mer) long peptide was synthesized from Genmed Synthesis Inc, U.S.A. The purity of the peptide was checked by HPLC. All other chemicals used in the study were of analytical grade purity. Cytokine ELISA kits were procured from Genzyme, U.S.A.

Proteolytic cleavage. The stock solution of chymotrypsin was prepared (10 mg/ml) in 1 mM HCl and stored at 4°C. Protein A (1 mg/0.5 ml PBS) was boiled for denaturation, equal amount (0.5 ml) of 0.4 M ammonium bicarbonate (pH 8.5) was added and finally chymotrypsin was added at the ratio of 50:1 (protein: chymotrypsin) to cleave the protein. The mixture was incubated at 37°C for 24 h for complete cleavage. Reaction mixture was boiled to terminate the reaction.

High-performance liquid chromatography. The chymotrypsin degraded product (peptides) of protein A, were separated by reverse phase HPLC. We applied the mixture of peptides to Nova-Pak C-18 (3.9 × 150 mm steel) column (Waters), using 0–100% gradient of 0.1% trifluoroacetic acid (TFA, Solvent A) and 60% acetonitrile in 0.1% TFA (Solvent B). Each peak was eluted from several runs, lyophilized and analyzed for amino acid composition.

The amino acid analysis of each fraction was done by PICO.TAG method (26), which involves three steps: (i) Hydrolysis of the peptide sample with HCl to yield free amino acid, (ii) precolumn derivatization of the sample with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids, and (iii) analysis of the above amino acids by Pico Tag column (Waters).

The molecular weight of the degraded product was determined by running through gel-filtration column, protein pak 60 (Waters, for molecular weight below 20 kD), along with the standard. We used gradient of 0.1 M Na_2HPO_4/NaH_2PO_4 (Solvent A) and 0.05 M Na_2HPO_4/NaH_2PO_4 (Solvent B).

Capillary electrophoresis. The 16-mer peptide suggested earlier by theoretical modeling studies as the best Fc binding peptide (24), also seems to be obtained by practical chymotrypsin degradation studies (Fig. 2) was synthesized. The IgG binding property of this 16-mer peptide was verified by immunoprecipitation followed by capillary electrophoresis. Protein A and normal human IgG (1:4) was incubated at 37°C for immunoprecipitation purposes. Similarly, the 16 amino acid synthetic peptide (EILHLPNLNEEQRNGF) was reacted with normal human IgG (20:1) and was incubated at 37°C for Immunoprecipitation (BSA was used as a negative control). The dose of protein A and peptide was selected from the dose response study (where precipitation occurs). The precipitate was dissolved in 0.2 ml phosphate buffered saline (pH 7.2) and used for Capillary electrophoresis (Beckman), along with the pure human IgG, peptide, protein A as controls. A Beckman P/ACE system 5010, equipped with an IBM PC (window-based control including system Gold software) was used in this study. Neutral capillary (Beckman), with an inner diameter of 50 μm and a total length 37 cm (UV detector at 30 cm). As an electrolyte a 20 mM phosphate buffer, pH 2.5, was employed. Samples were injected by 85-psi pressure.

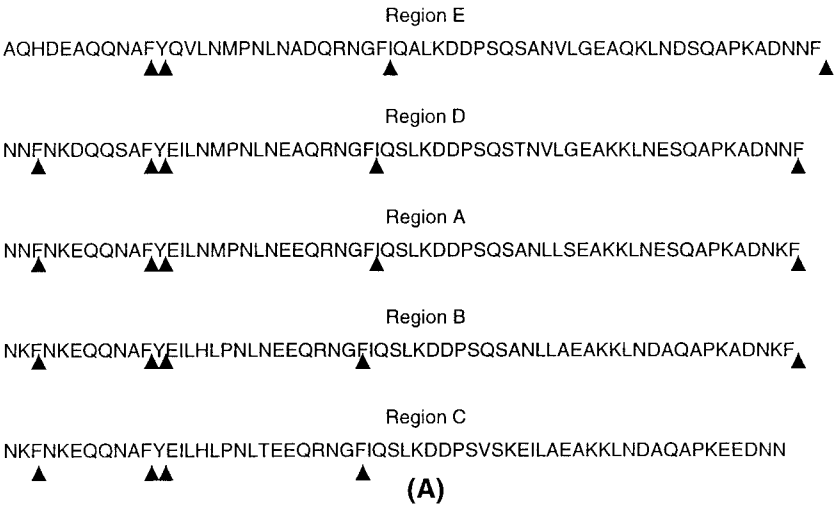
Animals. Random bred, Swiss albino mice (male, 15–20 g body wt) were obtained and kept in groups of 10 animals per polypropylene cage, under controlled temperature (20–25°C) and humidity (65–75%) with 12/12 h light/dark period. Animals were fed a synthetic pellet diet (Ashirvad Industries Ltd.) containing all essential nutrients, freshly obtained and stored under standard conditions, and water was given *ad libitum*.

Synthetic peptide or protein A treatment. Synthetic 16-mer peptide or protein A was administered intraperitoneally in 100 μl saline at a dose of 20 μg/animal or 1 μg/animal respectively, twice weekly for 2 weeks. The dose of peptide was selected from a dose response study where 20 μg peptide showed the IgG binding. Since 1 μg/animal dose of PA provides maximum immunoprotection and abrogation of chemical toxicity (8), this standard dose was used. The control animals received only saline in the same schedule. After the last injection blood was collected at different time intervals (2, 4, 6, 24, and 48 h). Five to seven mice were bleed for each time point, serum was separated and then pulled for each time point separately for cytokine analysis.

Cytokine assay. Enzyme-linked immunosorbent assay (ELISA) kits for TNFα and IL-1α were purchased from Genzyme, U.S.A., and the assays were done according to the manufacturer's protocol using the serum samples collected at different time point, after the last inoculation of the peptide or PA. The cytokine was estimated thrice, each time by performing separate set of experiments and representative of the results are shown.

RESULTS

Theoretical cleavage. Active regions (IgG binding) of different domains of PA are given in Fig. 1. Ideally, chymotrypsin cuts at Phe (F) and Tyr (Y) positions (Fig. 1A). Theoretical peptide map prediction on chymotrypsin degradation gives three major fragments, 8-, 16-, and 33-mer (approximately 0.8, 1.6, and 3.3 kD respectively, average 100 Da/residue) from each IgG binding regions and one long fragment (105 residues



(A)

Peptide	Calculated molecular wt.	Experimentally observed
N-terminal		
33 aa	3.3	3.2
16 aa	1.6	2.0
8 aa	0.8	1.0
C-terminal		
105 aa	10.5	-

(B)

FIG. 1. (A) Comparison of the IgG binding regions of PA. The sequences of the repetitive regions have been aligned to achieve maximum homology. The cleavage points for chymotrypsin are marked. (B) Theoretically predicted major fragments for chymotrypsin degradation and their calculated (100 Da/residue) approximate molecular weight (kD) matched with fragments derived from gel-filtration chromatography. Synthetic 16-mer, 20 mer (1.9 and 2.5 kD, respectively) along with few other synthetic peptides were used as standard.

approximately 10.5 kD) from cell-wall anchoring region (X-region) (Fig. 1B).

Molecular modeling study (25) qualitatively suggested that 16-mer peptide may retain Fc binding ability from interaction energy point of view. The 16-mer peptide from different domains are EILHLPNLNEEQRNGF (Domain B); EILNMPNLNEEQRNGF (Domain A); EILNMPNLNEAQRNGF (Domain D); EILHLPNLTEEQRNGF (Domain C); QVLNMPNLNADQRNGF (Domain E). P, N, and L at positions 6, 7, and 8 are conserved in all five domains fragment.

Release of protein A fragments by chymotrypsin digestion. We observed that chymotrypsin digestion of protein A molecule at 37°C for 24 h produced four fragments I, II, III and IV on reverse phase HPLC (Table 1). The amino acid analysis of each fragment shows absence of cysteine from all the fragments. Along with cysteine histidine, valine and methionine residues are absent from fragment I, isoleucine is absent in fragment II, histidine is absent in fragment III, histidine and arginine are absent in fragment IV. In the process of amino acid analysis asparagine and glutamine residues gets hydrolyzed to aspartic acid and glutamic acid respectively, therefore there is high content of aspartic acid and glutamic acid in all the fragments (I, II, III, and IV) (Table 1). In fragment I, leucine, isoleucine, serine, glycine, tyrosine and arginine are less whereas threonine alanine, phenylalanine, lysine and proline are approximately in equal amounts. Fragment II has less amount of tyrosine and valine whereas serine, glycine content are very high and histidine, arginine, threonine, alanine, methionine, leucine, phenylalanine, lysine, and proline residues are present in equal amounts. In fragment III there is high content of serine, isoleucine, leucine and phenylalanine residues whereas glycine, arginine, threonine, alanine, valine, lysine and proline are comparatively less whereas tyrosine and methionine are

TABLE 1

Amino Acid Residues (μmol/ml) in Chymotryptic Fragments of Protein A Separated by Reverse-Phase HPLC

	Amount in each fragment (μmol/ml)			
Amino acids	I	II	III	IV
Aspartic acid	0.1	0.8	0.1	0.1
Glutamic acid	0.02	0.06	0.2	0.1
Serine	0.001	0.5	0.1	0.02
Glycine	0.002	0.9	0.01	0.01
Histidine	—	0.2	—	—
Arginine	0.004	0.2	0.02	—
Threonine	0.01	0.1	0.02	0.04
Alanine	0.01	0.2	0.01	0.03
Tyrosine	0.002	0.01	0.002	0.01
Valine	—	0.02	0.02	0.01
Methionine	—	0.2	0.004	0.01
Isoleucine	0.0005	—	0.07	0.04
Leucine	0.0003	0.2	1.04	0.07
Phenylalanine	0.01	0.2	0.05	0.03
Lysine	0.01	0.2	0.04	0.07
Proline	0.007	0.2	0.02	0.02

Note. Each peak was eluted from several runs, lyophilized, and analyzed using the PICO.TAG method.

very less in amount. In fragment IV leucine and lysine are high whereas serine, glycine, threonine, alanine, tyrosine, valine, methionine, isoleucine, phenylalanine, and proline are comparatively less.

In gel-filtration column (Protein pak-60), we observed an overlapping peak on superimposing theoretically predicted synthetic 16-mer peptide (B-domain fragment) with chymotrypsin degraded protein A (Fig. 2). In this column chymotrypsin degraded protein A gave five peaks at retention time of 10.30, 12.03, 14.18, 15.86, and 18.88 minutes, corresponding to the molecular weight of approximately 5.8, 4.7, 3.2, 2.0, and 1.0 kD, respectively.

Immunoprecipitation and capillary electrophoresis. In capillary electrophoresis the standard PA, 16-mer peptide and human IgG gave a single peak at 10.2, 9.8, and 10 minutes. Whereas the complex of PA with IgG, 16-mer peptide with IgG gave peaks at 10.9 and 10.5 min, respectively (Figs. 3A and 3B).

Cytokines. TNFα and IL-1α were measured in the serum of mice treated with PA or 16-mer peptide. TNFα started increasing within 2 h of PA or peptide treatment and reached to its peak in 4 h with both the treatment but the increase was much higher in case of PA than that of 16-mer peptide (Fig. 3C). Thereafter the TNFα concentration was back to its control value at about 24 h. IL-1α also starts increasing within 2 h and reaches to its peak in 4 h (Fig. 3D) with PA. Whereas 16-mer peptide gives peak value in 24 h (Fig. 3D). In both the cases it returns to the basal level after 48 h.

DISCUSSION

The 16-mer peptide was derived from the theoretically predicted chymotrypsin cleavage studies, reflecting the sequence of B-domain among five homologous Fc binding regions of IgG (A, B, C, D, and E) (Fig. 1). We could get overlapping peak of 16-mer peptide with chymotrypsin degraded protein A on gel-filtration column (Fig. 2). Although the amino acid analysis (Table 1) of each chymotryptic fragment (I, II, III, and IV) shows slight deviation from theoretically identified 16-mer peptide, which is expected as there is slight variation in sequence of 16-mer peptide from different domains of PA (Fig. 1). We have also experimentally substantiated the IgG binding capacity of this 16-mer peptide by immunoprecipitation followed by capillary electrophoresis (Figs. 3A and 3B) and simultaneously described that the peptide upregulates the production of TNFα and IL-1α (Figs. 3C and 3D).

Protein A is a 395-residue-long protein having five homologous domains (with a sequentially different C-terminal end). Ideally chymotrypsin (Serine endopeptidase) cuts at C terminal end of Phe and Tyr. But it may cut in some other amino acid regions such as Leu, Met, Ala, Asp and Glu at lower rates. Taking Phe and Tyr cuts into consideration, we have predicted three fragments (approximately 1, 2, and 3 kD) each from IgG binding regions (Fig. 1) and one long fragment approximately 10.5 kD from cell wall anchoring region. In practical chymotrypsin digestion experiments, we could get an overlapping peak of 16-mer peptide (2 kD) with degraded protein A in gel filtration column (Fig. 2), along with four other peaks. Taking 20-mer (24) and 16-mer peptides of B domain (2.5 and

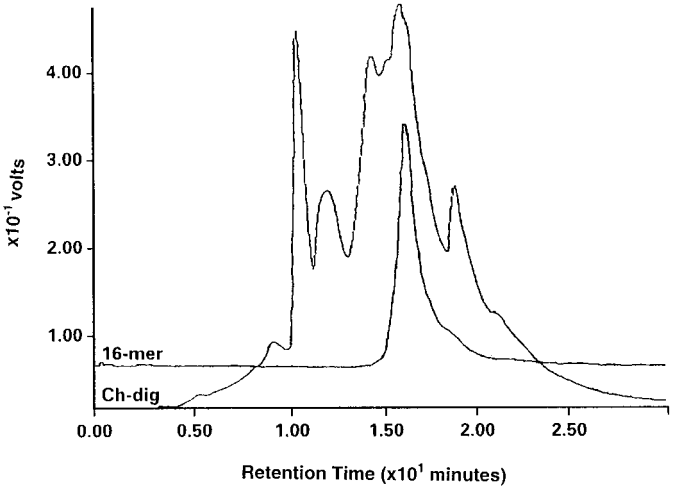


FIG. 2. Superimposed chromatogram of chymotrypsin degraded protein A (Ch-dig) and synthetic 16-mer peptide (16-mer) run through gel filtration column, protein pak 60, using gradient of 0.1 M Na_2HPO_4/NaH_2PO_4 (Solvent A) and 0.05 M Na_2HPO_4/NaH_2PO_4 (Solvent B).

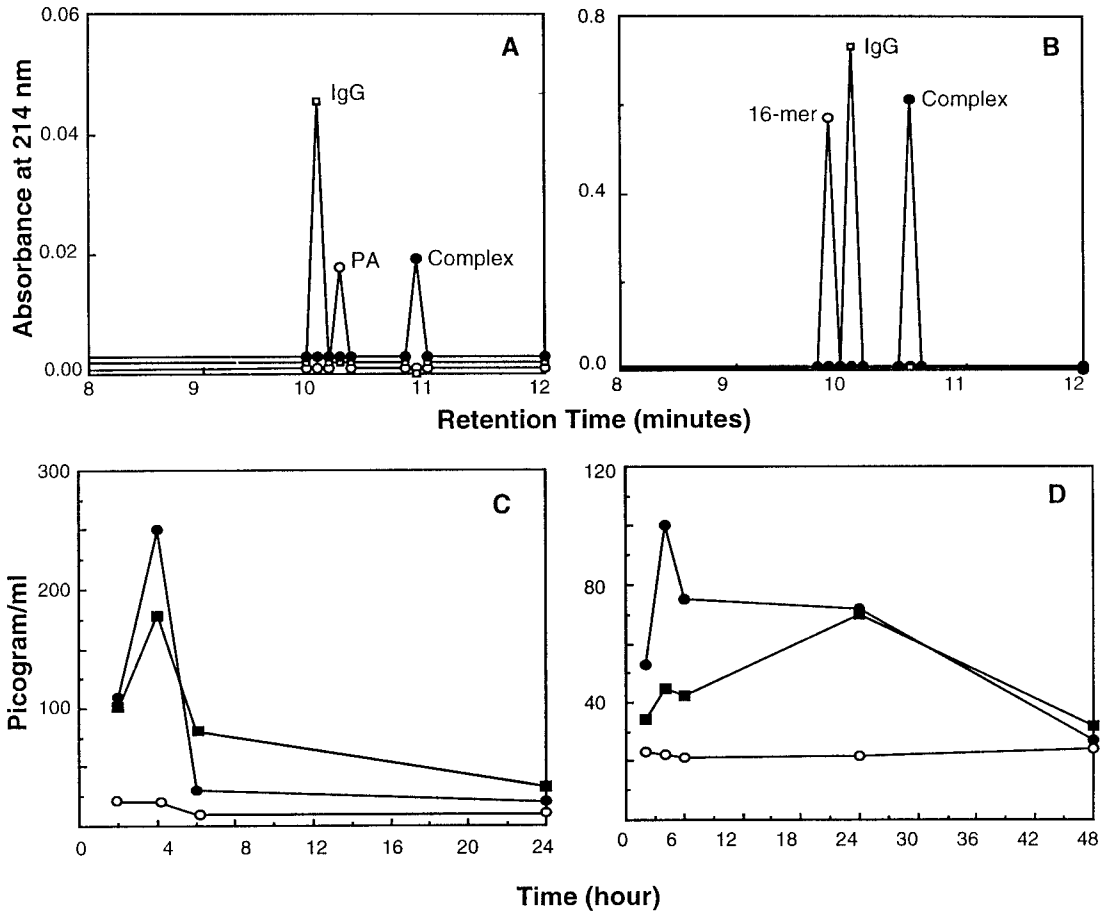


FIG. 3. Superimposed spectra of protein A (PA), peptide (16-mer), human IgG (IgG) and their complexes (complex) separated by neutral capillary electrophoresis. (A) Protein A with normal human γ-globulin (1:4) complex precipitate dissolved in 0.2 ml PBS superimposed with IgG and protein A (B) 16-mer synthetic peptide with normal human γ-globulin (20:1) complex precipitate dissolved in 0.2 ml PBS superimposed with IgG and 16-mer synthetic peptide. Effect of 1 μg PA/animal (●) or 20 μg 16-mer peptide/animal (■) and saline control (○) twice weekly for 2 weeks, to Swiss albino mice. Blood was collected at different time intervals after the last dose of PA or peptide. Picogram per milliliter of cytokines at different time points as determined by ELISA using Genzyme (U.S.A.) kits is shown (C) TNFα (D) IL-1α.

1.9 kD, respectively) and few other synthetic peptides as standard, molecular weight of these peaks were calculated qualitatively. 8 residue and 33 residue peptides (0.8 and 3.3 kD approximately) could be matched (Fig. 1B) with molecular weight of two of the practically obtained peaks (1 and 3.2 kD). The other C-terminal fragment of 105 residues (~10.5 kD) predicted theoretically might have been cleaved due to long digestion period and that may be the reason of obtaining 5.8 and 4.2 kD fragments in spite of single 10.5 kD fragment. All these three fragments (8-mer, 16-mer and 33-mer) from IgG binding regions of protein A could be formed from all five homologous domains. Amino acid sequences of fragments of different domains differ at only a few positions and thus making the molecular weight slightly different. It would be extremely difficult to separate them among themselves. The amino acid analysis (Table 1) of each chymotryptic fraction (I, II, III, and IV) does not exactly match to theoretically identified 16-mer peptide, which is expected as there is variation in sequence of 16-mer peptide from different domains of PA (Fig. 1).

Our results (Figs. 3A and 3B) established that PA as well as the synthetic peptide (16-mer) binds to the IgG. As a result of such binding there is a shift in retention time of each peak, when separated by capillary electrophoresis. When voltage is applied separation occurs due to combined action of electrophoretic migration and electroosmotic flow. It can be predicted from this study that 16-mer peptide may act as the appropriate chymotryptic sequence still retaining the Fc binding property.

In previously published literature there are a score of information regarding protein A and various of its biological properties many of which have been implicated as being initiated after protein A binds to IgG (27). Our observations described above (Figs. 3C and 3D) show that 16-mer peptide treatment results in production of TNFα and IL-1α (Figs. 3C and 3D) in mice serum. The induction of TNFα by 16-mer peptide

is highly significant. In an overview of phase II clinical trials, a variety of recombinant TNF preparations were infused to patients with breast cancer, advanced colorectal, gastric and renal carcinoma (28). The major problems associated with the exogenously administered TNF were the side effects of the high doses used. To overcome the short half life of TNF, continuous iv infusion was necessary, but the severe side effects remained. To limit the side effects, triggering of endogenous TNF was found more useful (28). It is already established that of TNF α and IL-1α act synergistically to eliminate tumor cells (29). In this connection, this 16-mer peptide mediated TNFα and IL-1α induction in mice appears very encouraging, with its future possible use in clinical cancer therapy.

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