Results
4.0 Results

4.1 Immunogenicity of HSA and cross reactivity of anti HSA antibodies

Human serum albumin used for the study was quite immunogenic and readily induced antibody formation in rabbits. Pre-immune and immunized antisera from 100 to 100,000 dilution was coated in the wells for titre determination. After blocking of the wells with skimmed milk, they were again incubated with the enzyme conjugate. The plates were then developed for the peroxidase activity. HSA was found to be highly immunogenic, as it gives a titre of 31,622 (Fig 4.1). Sharp precipitin lines were obtained on immunodiffusion of anti-HSA antiserum and the DEAE fraction against HSA (Fig 4.2 A). In Fig 4.2(B) the precipitin lines obtained show that antisera raised against HSA readily cross reacted with human serum but not with bovine serum.

4.2 Immunoaffinity layering

4.2.1 Immunodot analysis of HSA by immunoaffinity layering

Earlier studies from others (Alvarez-Kaza, 1993; Vanderberg, 1994) and from this laboratory (Farooqi et al., 1999) have shown that amounts of enzyme immobilized on a solid surface can be raised several folds by alternate incubations with the enzyme and anti-enzyme antibody. The strategy was described as immunoaffinity layering. It was envisaged that the strategy could be used to enhance the binding of secondary antibody-enzyme conjugate to the enzyme fixed on nitrocellulose and incubated with primary antibody. To enhance the sensitivity of the standard immunodot analysis procedure, immunoaffinity layering was performed with anti-HSA antibodies and the goat anti-rabbit IgG-HRP conjugate. HSA dissolved in 10 mM sodium phosphate buffer pH 7.4 was applied as spots on the nitrocellulose strips. A set of four strips prepared thus contained spots bearing 22.6 pg to 22.6 μg of HSA. The strips were blocked with skimmed milk in order to prevent non specific binding. They were then thoroughly washed with PBS-T buffer and incubated with anti-HSA-IgG for one hour.
Figure 4.1 Titre determination of the raised antisera by ELISA

Ninty six well microtitre plate was loaded with serially diluted antisera. After blocking with skimmed milk and washing with the wash buffer the plate was again incubated with the enzyme conjugate. The enzyme activity was read at 405 nm. For details see the text.
Figure 4.2 (A) Ouchterlony double diffusion of HSA against anti-HSA antiserum and IgG fractions isolated thereof.

Rabbits were immunized with HSA. The antiserum obtained was fractionated to isolate the β-globulin fraction as detailed in the text. The central well contained 30μg of HSA. Well a, 30μg of immunize serum; well b, 30μg of 20-40% ammonium sulphate cut and well c, 30μg of DEAE fraction.

(B) Ouchterlony double diffusion of anti-HSA-IgG against purified HSA, Human serum and BSA

Rabbits were immunized with HSA. The antiserum obtained was fractionated to isolate γ-globulin fraction as detailed in the text. The central well contained 50μl of antiserum. Well 1 and 2 had 30μg of BSA and HSA respectively and well 3 contained 50μl of human serum.
followed after thorough washing with the conjugate for the same duration. Strip I was
stained for the peroxidase activity while strips II, III and IV were again incubated,
after washing with buffer, with anti-HSA-IgG followed by the conjugate, prior to staining.
After an additional incubation with the primary antibody and the conjugate, strip II was
stained for the peroxidase activity. Strip III and IV were carried through additional one
and two incubation cycles respectively prior to staining for peroxidase activity.

Fig 4.3 shows the quantity of HSA readily detectable under standard conditions
used was 22,600 pg, although the sample bearing 2,260 pg of HSA was also visible as
a very faint spot. The intensity of the HSA spots increased remarkably and step-by-
step on further incubations with anti-HSA-IgG and the conjugate, and those bearing
far lower concentrations of HSA, not detectable under standard conditions, also became
visible. As evident from Fig 4.3, the HSA spot bearing 22.6 pg of the protein was also
readily detectable after four incubation cycles with anti-HSA-IgG and the conjugate.
While it was possible to further increase the sensitivity of the procedure by increasing
the number of the incubation cycles with anti-HSA-IgG and the enzyme conjugated
secondary antibody, this was accompanied by a marked increase in background staining.

4.2.2 Effect of time of incubation

Attempts were made to ascertain if the enhancement in the observed sensitivity
of detection of HSA with multiple incubation assays described in the previous experiment
were not merely related to the extended durations for which HSA was exposed to the
primary and secondary antibodies. For this purpose eight nitrocellulose strips each
bearing 22,600 pg, 2,260 pg, 226 pg and 22.6 pg of protein applied as spots a, b, c
and d were incubated for varying intervals with primary antibody followed by incubation
with the conjugate under standard conditions.

As evident from Fig 4.4 in the strip 1 incubated for 10 minutes with primary
antibody, only the spot bearing 22,600 pg of antigen was barely visible while the other
Figure 4.3  Immunodot analysis of HSA using the anti HSA antibody and HRP conjugate by multiple incubation assay.

Nitrocellulose strips bearing different HSA concentration were incubated with primary antibody and enzyme conjugate for layering process. Dot a, 22.6 pg; b, 226 pg; c, 2,260 pg; d, 22,600 pg of HSA. I, II, III and IV indicates the number of layers the strip is subjected to.
Figure 4.4  Effect of incubation time on immunodot assay.

Nitrocellulose strips bearing different antigen (HSA) concentrations were subjected to dot blot analysis. Dot a, b, c and d contained 22,600 pg, 2,260 pg, 226 pg and 22.6 pg of HSA respectively. Strips I, II III IV V VI and VII were subjected for 10 min, 15 min, 30 min, 1 hr, 4 hr, 8 hr and 16 hr respectively for each primary and secondary antibody incubation.
three spots containing 2,260 pg, 226 pg and 22.6 pg of HSA were not detectable. In strip II the intensity of the dots bearing 22,600 pg was somewhat increased, while strip III incubated for 30 minutes and then stained for peroxidase activity showed clear intensification of the spot with 22,600 pg of HSA and that containing 2,260 pg of HSA became clearly visible. In case of strip IV which was subjected to 1 hour incubation, the 22,600 pg spot of HSA became more intense accompanied by significant increase in the intensity of spot containing 2,260 pg of the antigen. Strip V, VI and VII that were incubated with the antibody for 4, 8 and 16 hours respectively showed no further improvement in the intensity of the spots. Longer incubations were also ineffective in rendering the 226 pg and 22.6 pg of HSA visible. Incubations beyond 8 hours in fact resulted in decrease in the intensity of the spots supporting the observation of Gershoni (1984) that antigen-antibody complex may dissociate after long incubations.

4.2.3 Immunodot analysis of HSA with intact anti HSA-IgG and F(ab)\textsubscript{2} derived thereof

In view of the probability of secondary antibody component of the conjugate recognizing the non-Fc regions of the primary antibody, it was envisaged that removal of the Fc portion of the latter may improve the binding process by decreasing the steric hindrance. Experiments were therefore undertaken to investigate the usefulness of the replacement of anti-HSA-IgG with the F(ab)\textsubscript{2} prepared thereof on the sensitivity of HSA detection by the multiple incubation procedure. Purified HSA was spotted on nitrocellulose strips and five spots of varying HSA concentrations were applied on two strips. While one strip was taken through four incubation cycles with anti-HSA-IgG followed by goat anti-rabbit IgG-HRP conjugate as described earlier, the other strip was subjected to the alternate incubations with anti-HSA- F(ab)\textsubscript{2} and the conjugate. Fig 4.5, shows a comparison of immunodot analysis of HSA using anti-HSA-IgG and F(ab)\textsubscript{2} derived thereof after four incubation cycles. The improvement in sensitivity as a result of substitution of F(ab)\textsubscript{2} (strip A) for IgG (strip B) was not very high but significant.
Figure 4.5  Immunodot analysis of HSA with intact anti-HSA-IgG and F(ab)\textsubscript{2} derived thereof.

Nitrocellulose strips bearing different HSA concentrations were subjected to immunoaffinity layering with intact anti-HSA-IgG (B) or F(ab)\textsubscript{2} derived thereof (A) and goat anti-rabbit-HRP conjugate after four incubation cycles. Dot a, 11.3 pg; b, 22.6 pg; c, 226 pg; d, 2,260 pg and e, 22,600 pg of HSA.
The spot bearing even 11.3 pg of HSA was clearly visible after four incubation cycle involving F(\(\text{ab}'\))\(_2\) and the intensities of the spots bearing higher concentration of the proteins increased when compared with those stained after multiple incubations using intact anti-HSA-IgG. Removal of the Fc region thus appears to facilitate a moderate enhancement in binding of secondary antibody conjugate, presumably by making antigen binding domains on F(\(\text{ab}'\))\(_2\) region more accessible.

### 4.2.4 Immunoaffinity layering using native and glutaraldehyde crosslinked anti HSA-IgG

An attempt to improve the sensitivity of detection was also made using anti-HSA-IgG crosslinked with glutaraldehyde. As shown in Fig 4.6, anti-HSA-IgG migrates as two bands corresponding to light and heavy chain and the IgG was transformed to high molecular weight adduct on treatment with gluteraldehyde. The preparation cross linked with 0.5% glutaraldehyde did not enter the gel on electrophoresis indicating the very high molecular weight nature of the adduct. Eight strips spotted with 15.6 pg, 156 pg, 1,560 pg and 15,600 pg of HSA as dots a, b, c and d respectively. Four were incubated with anti-HSA-IgG (Fig 4.7, Panel A) and four processed with glutaraldehyde crosslinked anti-HSA-IgG, (Fig 4.7, Panel B). In strip I, Panel (A) the dot bearing 15,600 pg of HSA is very clear while that with 1,560 pg is visible as a very faint spot. In contrast in strip I, Panel (B) even the dot bearing the 156 pg of antigen is clearly detectable. While the dots bearing 15,600 pg and 1,560 pg were also more intense as compared to Panel (A).

### 4.2.5 Immunodot analysis of HSA using affinity purified anti-HSA specific antibody

Experiments performed thus far were performed using the IgG fraction isolated from the immune sera. Specific antibodies against HSA were isolated by affinity purification using HSA coupled to CNBr activated Sepharose 4B following the published
Figure 4.6  Gel electrophoresis of glutaraldehyde treated anti-HSA-IgG

Anti-HSA-IgG was treated with glutaraldehyde and characterized using 10% acrylamide gel in presence of β-mercaptoethanol. The gel was stained and de-stained according to the procedure given in the text. Lane a and b contained 0.5% and 0.05% glutaraldehyde treated preparations. Lane c contained native anti-HSA-IgG.
Figure 4.7  Immunodot analysis of HSA by immunoaffinity layering using native anti HSA-IgG (A) and gluteraldehyde linked anti-HSA-IgG (B).

Strips of nitrocellulose membrane bearing 15.6 pg, 156 pg, 1,560 pg and 15,600 pg in dots a; b; c and d respectively. The strips were carried through One (I), two (II), three (III) and four (IV) incubation cycles as described in the text.
procedure (Stults, 1989). The preparation obtained thus contained 5mg/10ml of HSA. HSA specific antiserum was allowed to bind to the HSA-Sepharose 4B affinity column. The column was washed to remove any unbound IgG and the specific IgG eluted with 0.1 M glycine HCl buffer pH 3.5. These immunoaffinity purified anti-HSA antibodies were used to perform immunodot analysis of HSA. Four nitrocellulose strips were spotted with 8.2 pg, 82 pg, 820 pg and 8,200 pg of HSA as dots a, b, c and d respectively. The strips were first blocked with skimmed milk and then incubated with affinity purified anti-HSA-IgG for 1 hour and with the conjugate also for 1 hour at 37°C. Strip I was stained for the peroxidase activity, and strip II, III ans IV were taken through further incubation cycles (Fig 4.8). The dot bearing the 82 pg of antigen was very faint but detectable by the standard assay procedure. In the strip subjected to two cycles of incubation 82 pg spot could be seen clearly. The 8.2 pg spot was also visible in strip III as a faint spot. After four incubation cycles, 8.2 pg of HSA spot could also be clearly seen. The intensity of the spots also increased with each successive incubation cycle. The sensitivity of detection however appeared to improve only moderately when affinity purified antibodies were used instead of the IgG fraction.

4.2.6 Immunodot analysis of HSA in human serum using the multiple incubation assay

Four nitrocellulose strips were spotted with varying amounts of human serum as five dots. The strips were then taken through one, two, three and four incubation cycles, as described earlier in experiments, and Fig 4.9 shows the result of the study. As evident, HSA in the human serum sample bearing 3,944 pg of protein was barely detectable by the standard procedure. Successive alternate incubations as described earlier caused the appearance of new spots and marked intensification of those already visible. At the end of four incubation cycles, the spot of the human serum sample bearing 32 pg of protein was also detectable as a faint spot while that containing 39 pg protein turned more intense. Considering that albumin constitutes about half of protein
**Figure 4.8** Immunodot analysis of HSA using affinity purified anti HSA-IgG on nitrocellulose strips by multiple incubation assay.

Nitrocellulose strips bearing various concentration of HSA were carried through One (I), two (II), three (III) and four (IV) incubation cycles as described in the text. Dot a, 8.20 pg; b, 82 pg; c, 820 pg; d, 8,200 pg of HSA.
Figure 4.9  **Immunodot analysis of human serum by immunoaffinity layering.**

Nitrocellulose strips were spotted with whole human serum of different dilutions and subjected for four incubation cycles with anti-HSA-IgG and goat anti-rabbit-HRP conjugate. Dot a, 32 pg; b, 39 pg; c, 394 pg; d, 3,944 pg; e, 39,440 pg; of protein. I, II, III and IV indicates the number of layers the strips are subjected to.
in plasma, the sensitivity of its detection in plasma samples appears to be comparable with that of purified HSA.

4.2.7 Studies of albumin aggregation using multiple incubation assay

The applicability of the multiple incubation procedure to detect HSA aggregation at low concentrations was also investigated. HSA was subjected to 7.5% gel electrophoresis under non denaturing conditions (Fig 4.10). The lanes a, b, and c contained 5.6 ng, 56 ng, and 560 ng of albumin respectively. Subsequent to electrophoresis the protein bands were transferred on to nitrocellulose membrane according to the Towbin’s protocol (Towbin et al., 1979). Strips of panel I - IV were subjected to single, two, three or four incubation cycles respectively with primary antibody and the conjugate as described earlier in the text. As evident from Fig 4.10, panel I (a), aggregation is barely visible in the sample containing about 5.6 ng of HSA that migrated as a fast moving major band and a very faint band migrating about 75% of its distance, presumably representing the aggregated form of the protein. The slow migrating band was visible somewhat more prominently in the sample bearing 56 ng but was very clearly visible in that with 560 ng of protein, which also showed the presence of an additional far slower moving bands. Further incubations with primary antibody and secondary antibody conjugate however resulted in visualization of additional bands and at the end of the four incubation cycles, even the sample bearing 5.6 ng showed additional slow migrating bands. Evidently even at the lowest concentration of HSA used, formation of aggregates takes place, but the aggregates were apparently present at concentration below the limits of detection of the standard staining procedure. The aggregation could however be clearly visualized by the multiple incubation procedure and additional bands of various mobilities could be seen even in samples containing 5.6 ng of protein.
Figure 4.10  Multiple incubation assay showing albumin aggregation

HSA was subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Strips of panel I-IV were subjected respectively to single two, three and four incubation cycles with anti-HSA-IgG and conjugate. Lanes a; b and c contains 5.6 ng, 56 ng and 560 ng of protein.
4.2.8 Multiple incubation assay in ELISA

Wells of the polystyrene microtitre plates were coated with various HSA concentrations ranging between 0.0001 pg to 1 µg. They were blocked with 2% (w/v) skimmed milk, incubated with anti HSA-IgG for 1 hour at 37°C. This was followed by the incubation with goat anti-rabbit-HRP conjugate and estimation of the peroxidase activity colorimetrically. After four incubation cycles even 1 pg of antigen was detectable. Sensitivity appears to increase nearly 10 fold after four incubation cycles (Fig 4.11).

4.2.9 Multiple incubation assay in ELISA using F(ab)'2

The experiments described in section 4.2.8 was also performed using anti-HSA-F(ab)'2 instead of intact IgG. Plates were coated with varying amounts of HSA ranging from 0.0001 pg to 1 µg. Following the same standard procedure for layering, the plates were developed for peroxidase activity. Fig 4.12, shows the result after four incubation cycles. With F(ab)'2, 0.1 pg of antigen could be detected after four incubation cycles. This suggested significant improvement in sensitivity over the procedure that employed intact IgG.
Figure 4.11 Multiple incubation assay on ELISA plate

Ninety six well microtitre plate bearing HSA (0.0001pg to 1μg) were incubated with anti-HSA-IgG and enzyme conjugate through One (I), two (II), three (III) and four (IV) incubation cycles as described in the text.
Figure 4.12 Multiple incubation assay on ELISA plate using F(ab)\(_2\).

Ninty six well microtitre plate bearing HSA (0.0001pg to 1\(\mu\)g) were incubated with anti-HSA-F(ab)\(_2\) and enzyme conjugate for multiple incubation process. Results shown are of plates taken through four incubation cycles.
5.0 Modification of pepsin

5.1 Preparation of pepsin modified with histidine

Porcine pepsin contains 328 aminoacid residues, including two arginines, one histidine and one lysine. Our preliminary experiments indicated that affinity of pepsin for IDA Sepharose was only moderate, presumably due to the presence of the single histidine residue. The lysine residue protrudes from the surface behind the active site cleft on the C-terminal domain of porcine pepsin (Cottrell et al., 1995). It was therefore envisaged that additional histidine could be coupled to the side chain of lysine residue of the enzyme to facilitate stronger binding of the enzyme to the immobilized metal ion support.

In order to achieve covalent attachment of histidine, 6 mg of the aminoacid was mixed with 20-fold molar excess of glutaraldehyde in a total volume of one ml 0.02 M phosphate buffer pH 7.4 and incubated at room temperature for two hours. Amino group content of the glutaraldehyde treated histidine was determined with the help of TNBS method (Synder and Sobosinsta, 1975) and at the end of the incubation it was observed that greater than 95% of the amino groups were modified. In order to remove unreacted glutaraldehyde, the mixture was loaded on one ml column of Cu$^{2+}$-IDA Sepharose. The matrix was washed with distilled water and the bound histidine eluted with five ml of 20 mM EDTA. The eluted preparation of modified histidine was mixed with 3 mg of pepsin in sodium acetate buffer pH 5.5 in a total volume of 6.0 ml and the mixture incubated for three hours at 4°C. This mixture was again loaded on the Cu$^{2+}$-IDA Sepharose and the modified pepsin eluted with 20 mM EDTA. The eluted sample was extensively dialyzed against 200 mM sodium acetate buffer pH 3.5. The pepsin preparation retained 85% of enzyme activity after its treatment with glutaraldehyde treated histidine.
5.2 Properties of modified pepsin

5.2.1 Kinetic behaviour

5.2.1.1 Effect of substrate concentration

When pepsin catalyzed haemoglobin digestion was measured as a function of substrate concentration at 37°C in 0.2 M sodium acetate buffer pH 3.5, a typical Michaelis Menton behaviour was observed in both the cases of native and modified pepsin. The Line Weaver Burk plot indicated $K_m$ values for native and modified pepsin preparations as 200 mM and 222 mM, indicating only a minor alteration in the affinity of the enzyme for protein substrate as a result of the chemical modification. The $V_{max}$ of the modified pepsin remained nearly unchanged (Fig 5.1).

5.2.1.2 Effect of temperature

Temperature activity profiles for native and modified preparations were investigated in 0.2 M sodium acetate buffer, pH 3.5 and shown in Fig 5.2. No alteration in temperature maximum was observed and both native and modified pepsin showed maximum activity at 40°C. However the modified pepsin retained a relatively higher fraction of maximum enzyme activity at temperatures above 40°C.

5.2.1.3 Effect of pH

Activity profiles of native and modified pepsin were investigated in 0.2 M sodium acetate or sodium phosphate buffer at various pH at 40°C (Fig 5.3). The optimum pH value of the modified preparation was identical with that of the native enzyme.

5.2.2 Electrophoretic behaviour

The porcine pepsin modified as described above was dialyzed thoroughly against 0.2 M sodium acetate buffer, pH 3.5 and subjected to electrophoresis. Fig 5.4 (A)
Figure 5.1  Line Weaver Burk Plot for haemoglobin digestion by pepsin and modified pepsin preparations.

30 μg of pepsin and modified pepsin preparations were incubated in a series of tubes with the standard assay mixture containing varying concentration of substrate under standard conditions, and the activity was determined.
Native Pepsin

Modified pepsin
Figure 5.2  Temperature activity profiles of native and modified pepsin

Aliquots of pepsin and modified pepsin were incubated at indicated temperatures for 15 minutes and assayed for enzyme activity under standard conditions of pH and substrate concentration.

Figure 5.3  pH activity profiles of native and modified pepsin preparations.

Aliquots of native and modified pepsin preparation were incubated in 0.2 M sodium acetate (pH 3.0 - 5.0) or 0.2 M sodium phosphate (6.0 - 8.0) for 15 minutes and the activity determined under standard assay condition.
The graph above shows the % maximum activity of native and modified pepsin as a function of temperature (°C). The data points indicate that native pepsin has a higher % maximum activity compared to modified pepsin across the range of temperatures studied.

In contrast, the graph below illustrates the % maximum activity of native and modified pepsin as a function of pH. The graph suggests that native pepsin has a higher % maximum activity at lower pH values, whereas modified pepsin maintains a higher activity across a broader pH range.

**Temperature (°C):**
- Native pepsin: Increased activity from 20°C to 60°C, with a peak at approximately 40°C.
- Modified pepsin: Decreased activity from 20°C to 100°C.

**pH:**
- Native pepsin: Maintains activity from pH 2 to pH 6, with a peak at pH 4.
- Modified pepsin: Activity decreases from pH 2 to pH 10.
Figure 5.4  (A) Gel electrophoresis of native and modified pepsin.

Native and modified preparations were subjected to gel electrophoresis in 12.5% polyacrylamide in the presence of β-mercaptoethanol and SDS. Lane a, molecular weight markers; lane b, native pepsin (35μg); lane c, modified pepsin (35μg).

(B) Molecular weight determination of modified pepsin by Weber and Osborn procedure.

The relative mobility of the standard marker proteins from the SDS gel (Figure 5.4 A) were plotted against logarithm of molecular weight using least square analysis. Arrows indicate the position of the native and modified pepsin.
shows the electrophoretic behaviour of pepsin in reducing SDS PAGE. As evident from the Fig 5.4 (A) there was a very slight difference in the mobility of modified and native pepsin, with modified pepsin migrating slightly slower than native enzyme. The molecular weight of modified pepsin as calculated was 37.2 KDa and the native pepsin had a molecular weight of 35.8 KDa (Fig 5.4, B).

5.2.3 Autolysis of pepsin and modified pepsin

In order to compare the susceptibility to autolysis, native and modified pepsin in 0.2 M sodium acetate buffer, pH 3.5 were incubated for 18 and 24 hrs at 40° C. SDS-PAGE under reducing condition shows one major band for modified pepsin, and three in native preparation incubated for 18 hrs (Fig 5.5, lane d and e). Even after 24 hours the modified preparation showed only two major bands while native pepsin was cleaved to several fragments (Fig 5.5, lane b and c). A small band migrating slower than pepsin was observed in the modified pepsin preparation incubated for 24 hours.

5.2.4 Spectral studies

5.2.4.1 Ultra violet spectroscopy

The UV spectrum of native pepsin shows maximum absorbance at 277 nm, while modification resulted in slight alteration in the spectrum with a shift in the absorption maximum from 271 to 274 nm. There was observed a 3 nm blue shift with a decrease in the magnitude of absorbance (Fig 5.6).

5.2.4.2 Fluorescence spectroscopy

The histidine modified pepsin was also characterised for its fluorescence using an excitation wavelength of 280 nm over the range of 300 - 400 nm. As shown in Fig 5.7 the emission spectra obtained gave maximum intensity at 368 nm, both for native and modified pepsin preparation, but showed a decrease in the magnitude of fluorescence intensity in case of the modified preparation.
Figure 5.5 Autolysis of native and modified pepsin

Native or modified pepsin were subjected to SDS-polyacrylamide gel electrophoresis after their incubation for 18 or 24 hours at 40°C at pH 2. Lane a, molecular weight markers (98, 68, 43, 29, 20 and 14 KDa); lane b, modified pepsin after 24 hours incubation; lane c, native pepsin after 24 hours; lane d, native pepsin after 18 hours; lane e, modified pepsin after 18 hours; lane f, native pepsin at zero hour.
Figure 5.6  Ultraviolet spectra of native and modified pepsin

UV spectra of native and modified preparations in 0.2 M sodium acetate buffer were taken at pH 3.5. The protein concentration was 200 μg/ml and the pathlength was 1 cm.

Figure 5.7  Fluorescence spectra of native and modified pepsin

Fluorescence spectra of pepsin and modified pepsin in 0.2M sodium acetate buffer at pH 3.5. The protein concentration was 200 μg/ml. Samples were excited at 280 nm and band width was 10 nm.
5.2.4.3 Circular dichroism of native and modified pepsin

The far UV spectrum for native pepsin is characterized by the presence of a minima at 216 nm (Fig 5.8). Modification of pepsin with glutaraldehyde linked histidine resulted in only a marginal alterations in the spectrum leading to a slight dip at 219 nm.

The CD spectra in the near UV region reflects the contribution of aromatic side chains, disulphide bonds, and of prosthetic groups (Kuwajima, 1989; Dryden and Weir, 1991). Fig 5.9 shows the near UV-CD spectrum of pepsin in 250 - 300 nm range. Near UV-CD spectrum of native pepsin is characterized by a band at 258 nm and the modified pepsin also showed nearly similar spectrum. The small decrease in the MRE value indicates a slightly less ordered structure in case of the modified pepsin.

5.2.5 Binding of native and modified pepsin on Cu^{2+}-IDA Sepharose

Native or modified pepsin (2mg/ml) were mixed and incubated with Cu^{2+}-IDA Sepharose at increasing pH (2 - 8) for one hour. The matrix was centrifuged and the amount of protein in the supernatant was used to calculate percent binding. Fig 5.10 shows that at pH 2, both native and modified pepsin showed only about 19 % binding to IDA Sepharose. At pH 4, there was a slight increase in the binding of modified pepsin to IDA over native pepsin which shows only 20% binding. At pH 6 and 8 modified pepsin showed a marked increase in binding to the support over the native enzyme.

5.2.6 Elution of pepsin and modified pepsin bound to Cu^{2+}-IDA with EDTA

Elution profile of native and modified pepsin was studied at varying EDTA concentration. Native and modified pepsins were eluted with 5 ml of (4 - 24 mM) EDTA. Fig 5.11 shows that native pepsin elutes completely at 16 mM EDTA while modified pepsin elutes only 80 % under the condition. At 20 mM EDTA however both the preparations were eluted completely from the support.
Figure 5.8  Far UV-CD spectra of native and modified pepsin

Experiments were carried out at 0.2 M sodium acetate buffer pH 3.5 using 1 ml (0.35 mg/ml) of native (———) or modified pepsin (— — —) solution in a 1 cm path-length cuvette after two scans from 200 to 250 nm at room temperature under continuous nitrogen flush.
Figure 5.9  Near UV-CD spectra of native and modified pepsin

Spectra were generated using 1 ml (1.2 mg/ml) solution of native
(———) and modified pepsin (— — —) in a 1 cm path length cuvette
after two scans from 250 to 300 nm at room temperature under
continuous nitrogen flush.
Figure 5.10  Binding of native and modified pepsin to Cu^{2+}-IDA Sepharose

Native or modified pepsin was allowed to bind to Cu^{2+}-IDA at a pH of 2, 4, 6 and 8
The graph shows the percentage of binding for native pepsin and modified pepsin at different pH levels. The x-axis represents the pH levels ranging from 2 to 8, and the y-axis represents the percentage of binding ranging from 0 to 120.

At pH 2, both native and modified pepsin show low binding percentages. As the pH increases, the binding percentages increase. At pH 8, the binding percentage for modified pepsin is significantly higher compared to native pepsin.
Figure 5.11  Elution profile of native and modified pepsin from Cu^{2+}-IDA Sepharose

Native and modified pepsin bound to Cu^{2+}-IDA was eluted with increasing EDTA concentration.
The graph shows the effect of EDTA concentration on the % Elution of native pepsin and modified pepsin.

- **Native pepsin** is represented by a solid line.
- **Modified pepsin** is represented by a dashed line.

The x-axis represents EDTA concentration (mM) ranging from 0 to 30, while the y-axis shows % Elution ranging from 0 to 120.

As EDTA concentration increases, the % Elution for both native and modified pepsin increases. The % Elution for modified pepsin is consistently lower than that of native pepsin across all EDTA concentrations.
5.3 Purification and pepsinization of IgG and F(ab)'₂ formation.

5.3.1 Purification of IgG from anti-HSA antiserum

Commercial HSA (Sigma) is quite immunogenic in rabbits and readily elicited the formation of precipitating antibodies. Its higher titre generation is shown in Fig 4.10.

For the purification of anti-HSA antibodies, the procedure described by Catty and Raykundalia (1988) was followed. The antisera were precipitated between 20 - 40 % ammonium sulphate saturation, and the precipitate dissolved in 20 mM sodium phosphate buffer pH 7.2, was subjected to ion exchange chromatography on a DEAE cellulose column (1.8 x 10 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.2. Purification of IgG was followed by polyacrylamide gel electrophoresis performed in presence of SDS. Whole antiserum and ammonium sulphate fractions show bands corresponding to IgG and several other polypeptides (Fig 5.12, lane a and b). Nearly all other bands were removed after DEAE cellulose chromatography and the purified IgG preparation gave a single band (Fig 5.12, lane c) on electrophoresis.

5.3.2 Molecular weight of IgG

In order to determine the molecular weight of the isolated IgG preparation, SDS-PAGE was run along bovine serum albumin (68 KDa), ovalbumin (43 KDa), carbonic anhydrase (29 KDa), soyabean trypsin inhibitor (20 KDa) and lysozyme (14.2 KDa) as markers (Fig 5.13 A). The molecular weight of the polypeptides of IgG was calculated as per the procedure of Weber and Osborn (1969) by plotting the mobility of marker proteins Vs the logarithim of their molecular weights (Fig 5.13 B). The position of the migration of IgG polypeptides corresponded to their apparent molecular weights of 48 KDa and 25 KDa.
Figure 5.12  Purification of IgG

IgG was purified by salt fractionation and DEAE cellulose chromatography, and subjected to SDS-PAGE. Lane a, rabbit serum; Lane b, 20-40% ammonium sulphate fraction; Lane c, DEAE fraction.
Figure 5.13 (A) SDS-PAGE of IgG during various stages of purification

Antiserum, 20 - 40% ammonium sulphate fraction and fraction from DEAE cellulose and markers were incubated with sample buffer at 100°C for five minutes and subjected to 12.5% polyacrylamide gel electrophoresis in presence of SDS. Lane a, DEAE cellulose fraction; lane b, 20 - 40% ammonium sulphate fraction; lane c, antiserum; lane d, molecular weight markers.

(B) Molecular weight determination by Weber and Osborn procedure

The relative mobility of the standard marker proteins from SDS gel (5.13 A) was plotted against logarithm of molecular weight using least square analysis. Arrows indicate the positions of the large and small molecular weight peptides from the DEAE fraction.
5.3.3 Cleavage of IgG with modified pepsin and purification of F(ab)'

The IgG fraction obtained by ammonium sulphate fractionation and DEAE cellulose chromatography was subjected to proteolysis with the modified pepsin preparation to obtain F(ab)'\textsubscript{2} fragments. Initially, the procedure described by Nisonoff \textit{et al} (1960) for IgG cleavage by pepsin was followed. Two mg/ml of IgG solution was incubated with modified pepsin in a ratio of (1:100) at 40°C for 24 hours at pH of 4.5. The reaction was terminated by adding tris salt that increased the pH to 8.

Fragmentation of the IgG was studied by SDS PAGE under reducing conditions. The gel shows the disappearance of the band corresponding to IgG with a significant increase in the band of higher mobility (Fig 5.14, lane b) suggesting the cleavage of IgG into F(ab)'\textsubscript{2}/Fc fragments. The faster moving protein bands presumably correspond to degraded Fc fragment.

With a view to achieve the separation of F(ab)'\textsubscript{2} generated from intact IgG/Fc, IMAC was used. Fig 5.14, lane a shows a single band with mobility corresponding to IgG inferring the complete binding of intact IgG, Fc and fragmented Fc to Cu\textsuperscript{2+}-IDA Sepharose.

Optimum conditions of IgG cleavage by modified pepsin, were evaluated by studying the dependence of the process on temperature, pH and time of incubation.

5.3.3.1 pH dependent cleavage of IgG

IgG (2 mg/ml) cleavage with modified pepsin preparation was investigated after 12 hours incubation at 40°C at various pH values between (3.5 to 5.0). The reaction was stopped by the addition of 0.2 M NaOH. Fig 5.15 shows that IgG was best cleaved at pH 3.5 while at pH 5.0 there was very little cleavage of IgG. At pH 4.0, most of IgG was cleaved leaving only a faint band corresponding to heavy chain.
Figure 5.14 Pepsinization of IgG

2 mg/ml IgG was exposed to pepsin at pH 4.5 at 37°C. The reaction was terminated after 24 hours by the addition of tris salt. Fragmented IgG and F(ab')₂ obtained was subjected to non reducing PAGE. Lane a, supernatant obtained after binding of the pepsin digested IgG on Cu²⁺-IDA Sepharose Intact IgG; Lane b, IgG treated with pepsin; Lane c, intact IgG.
Figure 5.15  pH dependent cleavage of IgG

Pepsin digestion of the IgG was carried out at various pH values and the digest subjected to SDS-PAGE. Lane a, intact IgG; lane b; c; d and e, pepsin digests prepared at pH 5.0, 4.5, 4.0, 3.5 respectively.
5.3.3.2 Temperature dependent cleavage of IgG

IgG was cleaved with the modified pepsin preparation at pH 3.5, for 12 hours at various temperatures between 4°C to 40°C. Fig 5.16, lane b shows that the fragmentation of IgG was barely detectable at 4°C, while at 20°C cleavage was quite significant (Fig 5.16, lane c). Presence of a single band in the preparation exposed to protease at 40°C shows complete fragmentation of IgG (Fig 5.16, lane d).

5.3.3.3 Time dependent cleavage of IgG

IgG was incubated with modified pepsin preparation at pH 3.5 at various time intervals at 40°C, the reaction was stopped by adjusting the pH to 8 with 0.2 M NaOH and the reaction products characterized by SDS-PAGE. Fig 5.17, lane d shows that after one hour incubation nearly all the IgG was cleaved.

5.4 Binding of F(ab) monomer to Cu²⁺-IDA Sepharose

Experiments were performed to determine if the F(ab) monomers bind to IDA Sepharose. This was considered important since small amounts of the monomer may be formed during the proteolysis of IgG to F(ab)²⁺. For the purpose 50 µg of each F(ab)²⁺ and F(ab) obtained as described in methods were mixed and loaded on Cu²⁺-IDA Sepharose, and the unbound fraction was characterized by PAGE. Fig 5.18, lane b shows 2 bands corresponding to F(ab)²⁺ and Fab, lane a had only one band corresponding to F(ab)²⁺ indicating essentially complete binding of F(ab) monomer to the Cu²⁺-IDA Sepharose.

5.5 Isolation of F(ab)²⁺ from unfractionated serum

Five hundred microlitres of serum was adjusted to pH 3.5 with the help of 0.06 N HCl, digested with 500 µg of modified pepsin at 40°C. After one hour incubation, the reaction was terminated with 0.2 N NaOH. One hundred µl of digested serum was loaded on 300 µl of IDA Sepharose loaded with Cu²⁺, Fe²⁺, Ni²⁺, Co²⁺ or
Figure 5.16 Temperature dependent cleavage of IgG

SDS-PAGE of the pepsin digest of IgG carried out at various temperatures, assessed under reducing conditions. Lane a, Intact IgG; Lane b; c and d has IgG digested at 4°C, 20°C, 40°C respectively. Lane e, marker proteins (98,68,43,29,20 and 14 KDa).
Figure 5.17  Time dependent cleavage of IgG

IgG digested with pepsin for various time intervals was subjected to SDS-PAGE. Lane a, marker proteins (98, 68, 43, 29, 20 and 14 KDa); lane b, intact IgG; lane c; d; e; f and g pepsin digests obtained after 30 minutes, 1 hour, 4 hours, 8 hours and 12 hours respectively.
Figure 5.18  Binding of F(ab) monomer to Cu\textsuperscript{2+}-IDA.

A mixture of F(ab)\textsubscript{2} and F(ab) prepared as described in methods was incubated with Cu\textsuperscript{2+}-IDA-Sepharose. The unbound fraction was subjected to 7.5% non-reducing PAGE. Lane a, IDA supernatant; Lane b, mixture of F(ab)\textsubscript{2} dimer and F(ab) monomer.
Figure 5.19 SDS-PAGE of serum digested with modified pepsin and binding to IDA-Sepharose loaded with various metal ions

The serum digest as described in the text was allowed to bind to Cu²⁺-, Fe²⁺-, Ni²⁺- and Zn²⁺- IDA Sepharose. Unbound fraction was subjected to PAGE in absence of β-mercaptoethanol (Panel A) or in presence of β-mercaptoethanol (Panel B).

Panel A, lane a, pepsin digest, lane b,c,d,e, and f had the supernatant of Fe²⁺-, Cu²⁺-, Co²⁺-, Ni²⁺- and Zn²⁺- chelated IDA Sepharose.

Panel B, lane a, pepsin digest, lane b,c,d,e and f contained the supernatant of Zn²⁺-, Ni²⁺-, Fe²⁺-, Co²⁺- and Cu²⁺- chelated IDA Sepharose.
Figure 5.20 High performance gel filtration chromatography of IgG and its pepsin digest.

Rabbit IgG was digested with pepsin and the digest incubated with Cu$^{2+}$-IDA Sepharose. The samples were analyzed using a Protein Pak ® column. (A) Intact IgG, (B) Pepsin digest of IgG, (C) supernatant of IgG digest incubated with Cu$^{2+}$-IDA Sepharose.
Figure 5.21  High performance gel filtration chromatography of serum and its pepsin digest.

Rabbit serum was digested with pepsin and the digest incubated with Cu^{2+}-IDA Sepharose. The samples were analyzed using a Protein Pak\textsuperscript{®} column. (A) serum, (B) pepsin digested serum proteins, (C) supernatant of serum digest incubated with Cu^{2+}-IDA
terminated by adjusting the pH of the reaction mixture to 8 with the help of 20 μl of 0.2 M NaOH. The sample was centrifuged and the supernatant analyzed both by reducing and non-reducing SDS-PAGE. Fig 5.22 panel (A), lane b, c and Fig 5.22 panel (B), lane a, b shows a single band indicating the formation of F(ab)'2.

5.7.1 Isolation of F(ab)'2 in a single tube from various mammalian species

IgG was isolated from the sera of sheep, goat, buffalo, rabbit and rat. Prior to incubation with the reaction mixture, the pH of the IgG was adjusted to 3.5. The IgG solution (1 mg/ml) from each species was added to the tube, 0.2 M NaOH was added to stop the reaction after one hour incubation at 40°C. Supernatant collected was run on SDS-PAGE. IgG from all the species showed the presence of only F(ab)'2 in the unbound fraction (Fig 5.23). All the lanes give a single band confirming their homogeneity.

5.8 Antigenicity of F(ab)'2 isolated from IgG

Immunodiffusion was also performed to check the antigen binding affinity of F(ab)'2 preparation. Sharp precipitin lines were obtained on immunodiffusion of anti HSA- F(ab)'2, and affinity purified anti-HSA-F(ab)'2 prepared from the above procedure indicating the homogeneity of the preparation (Fig 5.24).
Figure 5.22 Production of purified F(ab)² from IgG in single tube.

1 mg/ml IgG was incubated with modified pepsin, IDA-Sepharose and CuCl₂ at pH 3.5 for 1 hour unbound fraction was subjected to SDS-PAGE under reducing condition (Panel A), non reducing condition (Panel B).

Panel A, lane a, marker proteins (98, 68, 43, 29, 20 and 14 KDa); lane b and c contains 40 and 80 µl of the unbound fraction. Panel B, lane a and b had 80 and 40 µl of unbound fraction respectively.
Figure 5.23  Preparation of F(ab)'2 from IgG derived from various mammals

IgG derived from various mammalian sera were digested with modified pepsin and the reaction mixture purified on Cu(II)-IDA Sepharose. The supernatant was subjected to SDS-PAGE along with β-mercaptoethanol as described in the text. Lane a; b; c; d and e had 50 μl of fraction that did not bind to IDA Sepharose from sheep, goat, buffalo, rabbit and rat respectively.
Figure 5.24  Ouchterlony double diffusion of anti-HSA-IgG and anti-HSA-IgG-F(ab)\'_2 against HSA.

Rabbits were immunized with HSA antiserum obtained was fractionated to isolate the whole \(\gamma\)-globulin and anti HSA IgG fraction (affinity purified) as detailed in the text. IgG obtained was treated with modified pepsin preparation to yield F(ab)\'_2 in the single tube incubation procedure. The central well contained 30\(\mu\)g of HSA; well 1, anti-HSA antiserum; well 2, anti-HSA-IgG; well 3, F(ab)\'_2 derived thereof; well 4 and 5 had anti-HSA specific IgG and F(ab)\'_2 derived thereof respectively.