In-Vitro Stability of Human Alpha-Fetoprotein

James T. Wu and Joseph A. Knight

We assessed the stability of alpha-fetoprotein (AFP) in clinical specimens in the presence and absence of serum and albumin, at different temperatures and concentrations. We find it depends on both AFP concentration and incubation temperature. Dilution of most specimens with either phosphate buffer or phosphate-buffered saline or by immunoelectrodiffusion resulted in some loss of AFP. Attempts to stabilize AFP during either sample dilution or incubation by use of albumin in concentrations up to 1 g/L did not protect it from inactivation unless normal human serum was also included. Frozen AFP solutions were less stable than solutions stored at 4 °C. AFP was most stable when lyophilized and stored desiccated. The AFP-inactivation curves were usually nonlinear. Apparently both polymerization and degradation occur simultaneously as AFP loses its activity. Proteolytic enzyme inhibitor and sulfhydryl reagent not only failed to protect it from inactivation, they appeared to speed it.

Additional Keyphrases: amniotic fluid · storage conditions · anti-denaturants

Measurement of alpha-fetoprotein (AFP) in both amniotic fluid and maternal serum is useful in the prenatal diagnosis of neural tube defects (1). In addition, data on its concentration are helpful in the diagnosis and management of patients with primary hepatoma, yolk sac carcinoma, and teratoblastoma (2). The stability of AFP in clinical specimens has never been questioned, possibly because early techniques were so insensitive. However, AFP is now measured in nanogram amounts by radioimmunoassay (RIA), enzyme immunoassay (EIA), or immunoelectrodiffusion (IED). As a result, information on the stability of AFP becomes essential, particularly when dilution is required before assay. Moreover, use of the proper diluent is also important to prevent inactivation during sample dilution.

We reported previously (3) that during isolation and purification of AFP, it both polymerized and became inactivated. Additional information regarding the stability of AFP at various stages of its purification will further facilitate the production of pure AFP. Moreover, it is equally important to establish optimal conditions for its storage; for example, in our experience, freezing does not prevent AFP from deterioration.

Materials and Methods

Materials

Phenylmethylsulfonyl fluoride, a protease inhibitor, was from Sigma Chemical Co., St. Louis, MO 63178. Agarose powder (low electrophoresis value), reagents for polyacrylamide gel electrophoresis, and a silver-reagent kit were from Bio-Rad Laboratories, Richmond, CA 94804.

Anti-AFP was from DACO, Santa Barbara, CA 93101, and lactoperoxidase (EC 1.11.1.7) from Calbiochem, San Diego, CA 92121. Sepharose 6B was from Pharmacia Fine Chemicals, Piscataway, NJ 08854. AFP used for stability studies was isolated from human cord serum (4, 5). The purity of the original preparation exceeded 95%. Because the AFP preparation had been stored frozen for about a year, it contained a small amount of polymer and degradation product, but less than 10 g of serum albumin per kilogram of total protein.

Stability Studies

To study AFP stability, we prepared and stored an AFP stock solution (>100 µg/mL) at 4 °C. At various times, aliquots were diluted, sealed in separate test tubes, and incubated in a water bath at a fixed temperature. Analysis for AFP was usually performed at the same time at the end of the incubation period. In most experiments, we measured AFP by IED, so that most specimens needed no dilution before assay.

Miscellaneous Methods

IED. Plates were prepared by coating 10 g/L agarose gel containing either 30 µL or 100 µL of DACO anti-AFP per 30 mL of gel in 0.2 mol/L barbital buffer; the gel thickness was 1.2 mm. With such plates AFP concentrations of 0.1 to 5 mg/L could be measured directly. Electrophoresis was usually carried out at 20 V/cm for 2 h, cooling with tap-water.

Column chromatography on Sepharose 6B. 125I-labeled pure AFP was chromatographed on a 1.5 x 85 cm column containing Sepharose 6B after incubation, as illustrated below in Figure 5. We used phosphate buffer (10 mmol/L, pH 6.8) for column equilibration and for elution, collecting fractions of about 7 mL per tube. The radioactivity in each tube was counted directly in a gamma counter.

Radioiodination of AFP. This iodination involved lactoperoxidase. Briefly, we mixed 20 µL of AFP solution in 50 mmol/L sodium acetate, pH 5, with 5 µL (0.1 U) of enzyme, 10 µL (1 mCi) of Na125I, 10 µL of a 0.1 mmol/L solution of acetic acid, and an additional 60 µL of the sodium acetate buffer. The reaction was initiated by adding 10 µL of a freshly prepared 100 mg/L solution of hydrogen peroxide. After 10 min at room temperature, we added an additional 10 µL of 100 mg/L hydrogen peroxide solution. The reaction was stopped 5 min later by adding 150 µL of a 500 mg/L solution of sodium metabisulfite and 300 µL of a 30 g/L solution of potassium iodide. Residual free 125I was removed either by dialysis or by passing it through a column of G-25 Sephadex.

Results

Stability of Pure AFP

We examined the stability of AFP solutions in the presence of other proteins at -20, -60, 4, 25, and 30 °C, with AFP concentrations ranging from 0.1 to 50 mg/L (Figures 1 and 2). As expected, AFP stability was both temperature- and concentration-dependent. For example, at 0.24 mg/L, AFP was adequately stable at 4 °C for only a week (Figure

---

1. Nonstandard abbreviations: EIA, enzyme immunoassay; IED, immunoelectrodiffusion; AFP, alpha-fetoprotein.
2. Received March 13, 1985; accepted June 3, 1985.
Fig. 1. Stability of AFP at various concentrations and temperatures
AFP, 50 g/L, was diluted 50-fold before assay. We used phosphate buffer, pH 7.4 to dissolve AFP and for dilution. Other concentrations were assayed by IED without dilution. AFP concentrations were 50 (□), 1 (○), 0.5 (●), 0.24 (△), and 0.1 (Δ) mg/L. Normal human serum (NHS) was added to some samples before freezing but not (X) to others.

Fig. 2. AFP stability curve
Results of three separate experiments (Δ, ○, ●) were combined. AFP, 10 mg/L, was incubated at 30 °C and was measured by IED at various time intervals

1), but for at least a month at 4 °C when the concentration was 50 mg/L (data not shown). On the other hand, from the AFP stability shown (Figure 2) at 1 to 50 mg/L, AFP should be adequately stable at 10 mg/L for a few days at 25 °C but only a few hours at 30 °C. To our surprise, pure AFP was less stable when stored frozen at either -20 or -60 °C (Figure 1), even though we took care not to freeze or thaw the AFP rapidly. Because AFP loses activity at an inconstant rate, it was difficult to estimate the rate under any specific condition. It was also difficult to reproduce the results exactly in repeated experiments. The finding of an initial lag phase of inactivation followed by a rapid acceleration rate was unexpected. As a result, evaluating a complete inactivation curve for any specific temperature and AFP concentration required several experiments.

Effect of Serum Proteins
In an attempt to stabilize AFP, we added serum albumin to the AFP solution. Neither bovine nor human serum albumin stabilized AFP (Figures 3, 4). Indeed, AFP became less stable when incubated with increasing bovine serum albumin concentration (Figure 4). On the other hand, when AFP was incubated longer (e.g., 19 days) at 22 °C, it was more stable in the presence of higher concentrations of bovine albumin (Figure 4A). Similar results can be seen (Figure 3) at 30 °C: AFP stability increased in the presence of either bovine or human serum albumin, the latter appearing to be slightly more effective than the former at the same protein concentrations. The reasons for these observations are not clear.

On the other hand, normal human serum was the most effective agent in protecting AFP from inactivation (Figures 1, 3, 5); 10 to 100 mL/L (protein concentrations approximate...
ly 0.5 to 5 g/L) considerably slowed AFP inactivation. Because almost two-thirds of the protein in serum is albumin, it is not immediately obvious why serum was more effective than albumin.

We noted this same stabilizing effect of added protein in clinical specimens such as serum and amniotic fluid (data not shown). We found that AFP in both undiluted serum and amniotic fluid was stable for more than a month at room temperature and 30°C, but in the diluted specimens was not. Cord serum diluted 50-fold and amniotic fluid diluted 10-fold with phosphate-buffered isotonic saline lost their AFP activity within a few weeks.

Effect of Lyophilization

Although polymerization reportedly (2) may occur during lyophilization, we found this method to be the most effective in hindering denaturation of AFP during storage: no AFP activity was lost when lyophilized AFP was incubated at temperatures up to 30°C for six days (Figure 3). Our studies showed that polymerization during lyophilization was dependent on AFP concentration. When the AFP concentration exceeded 100 mg/L during freezing before lyophilization, no polymer was formed.

Effect of Sample Dilution

With most current quantitative techniques, one can measure AFP in nanogram amounts; therefore, the sample usually must be diluted before assay. However, dilution of relatively pure AFP results in some AFP loss: e.g., the initial rapid loss of AFP in Figure 6 due to dilution. Use of the less-sensitive IED technique alleviated the problem somewhat because less dilution was required than with RIA. To test the effect of dilution on AFP stability, and to establish the optimal concentration of human serum in the buffer, we tested AFP stability at four different AFP concentrations, diluting samples with four different buffers immediately before IED (Figure 7). Apparently, phosphate-buffered saline containing 10 mL of serum per liter most effectively protected AFP from denaturation, regardless of the final AFP concentration. On the other hand, dilution of AFP with phosphate buffer or phosphate-buffered saline in the absence of normal human serum resulted in the loss of AFP activity. The extent of inactivation also depended on the final AFP concentration, with no differences between phosphate buffer and phosphate-buffered saline as diluent.

Effect of Sulfhydryl Reagent and Proteolytic Enzyme Inhibitor

During incubation, AFP loses activity as a result of both polymerization and degradation. The less-active AFP polymer (3) may be produced by a mechanism similar to that for polymeric serum albumin (7): formation of disulfide bonds between sulfhydryl groups of monomeric AFP, followed by the disulfide exchange reaction. Loss of AFP activity may also be ascribed to the presence of trace amounts of proteolytic enzyme, which could more or less degrade AFP during incubation. Therefore we added both a sulfhydryl reagent, dithioerythritol, and a proteolytic enzyme inhibitor, phenylmethylsulfonyl fluoride, to test whether they could prevent the loss of AFP activity.

Pure 125I-labeled AFP was diluted to about 25 mg/L with 10 mmol/L phosphate buffer, pH 7.4: (a) in the absence of other additives, (b) in the presence of 2 g of phenylmethylsulfonyl fluoride per liter, and (c) in the presence of 10 mmol of dithioerythritol per liter. As shown in Figure 8A, AFP slowly lost its activity when incubated alone; surprisingly, addition of dithioerythritol or phenylmethylsulfonyl fluoride not only failed to stabilize AFP, each actually speeded its inactivation. The most striking effect was in the presence of dithioerythritol: all AFP activity was lost after 12 h at 30°C.

AFP Polymerization and Degradation

To determine what molecular changes occur in AFP during inactivation, we took aliquots of the AFP solution at various intervals during incubation and studied them by
both polyacrylamide gel electrophoresis (Figure 8C) and gel filtration chromatography (Figure 8B). We demonstrated previously (3) that AFP polymers were produced during AFP isolation, and that their presence could be detected by polyacrylamide gel electrophoresis. However, the use of column chromatography on Sepharose 6B not only confirms the existence of polymers, it also allows estimation of the amount of degradation product present. Because we used only pure AFP in these experiments, any protein bands revealed by the electrophoresis must be derived from AFP. In Figure 8C, the polyacrylamide gel pattern clearly demonstrates that AFP was polymerized at the same time it lost its activity. As incubation continued, the bands corresponding to the dimer became more intensified. Bands corresponding to higher polymers also appeared as the monomeric bands gradually faded. In addition, bands moving ahead of AFP monomer (corresponding to AFP degradation products) increased in intensity with time. However, at the end of the incubation period the intensities of all bands were diminished. Therefore, we believe that all AFP molecules were eventually degraded into small peptides and thus became no longer detectable by polyacrylamide gel electrophoresis.

The AFP polyacrylamide gel patterns (2C, 2G, 2H in Figure 8C) indicate that there was also polymerization and degradation in the presence of phenylmethylsulfonyl fluoride. Comparing 2C, 2G, and 2H with 1A (which represents the AFP pattern before incubation) shows that AFP monomer was concurrently lost. These observations are also supported by the activity profile shown in Figure 8A, which shows that phenylmethylsulfonyl fluoride did not protect AFP from either inactivation or degradation.

The most unexpected finding was the gel-electrophoresis pattern of AFP incubated in the presence of dithioerythritol. Here, all AFP molecules were degraded into smaller peptides (3C, 3G, and 3H in Figure 8C). This decrease in band intensity from 3C to 3H indicates that the concentration of the degradation products of AFP continued to change with time. The more diffuse band (3H) could be due to a mixture of degradation products of similar size, an observation supported by the elution profile shown in Figure 8B. However, only the radioactivity, not the peptide content, was monitored after column chromatography on Sepharose 6B. Some degradation products might not have included the \(^{125}\)I label, and so would not have been detected. Nevertheless, changes in the shape and peak height of the middle (indicating the amount and ratio of monomer to dimer present at a specific time) and last peak (appearing at total volume, \(V_t\), indicating the amount of degradation product present) suggest that the loss of AFP monomer and the increase in both AFP dimer and degradation products probably occurred simultaneously.

The relatively high radioactivity detected at the trough between the middle and last peaks (I(G, Figure 8B) also suggests that degradation products of various sizes may be present. The position and shape of peaks 2G, at the lower portion of Figure 8B, indicates that most of the AFP incubated in the presence of inhibitor was degraded; very little monomer and dimer were present. Therefore, the monomer and dimer shown by polyacrylamide gel electrophoresis may represent only a small portion of the total AFP remaining in solution at that time. On the other hand, the appearance of peak 3G (Figure 8B) is in complete agreement with the results shown by polyacrylamide gel electrophoresis. It also suggests that no AFP dimer or monomer was present after AFP was incubated with dithioerythritol.

**Discussion**

The finding that AFP was unstable in the presence of serum albumin was unexpected. Although we are still uncertain about the nature of this effect, we believe that it might be related to the binding of free fatty acids by serum albumin, a phenomenon known to affect its stability (7, 9). Bound fatty acids can be transferred in solution between albumin and AFP (10). Therefore, it is likely that fatty acids originally bound to AFP were removed during incubation by added albumin. Once the transfer of fatty acids was completed (or had reached equilibrium), the stability of the remaining AFP would be affected by the concentration of added proteins. As demonstrated in Figure 4, AFP stability increases with increasing albumin concentration.

AFP probably undergoes various molecular rearrangements simultaneously during the course of inactivation. AFP monomers may initially be converted to dimers and then to higher polymers. Because the dimers have half the original activity of the monomers (3), and because only a small proportion of monomers would have been converted at the beginning of incubation, no appreciable loss of overall AFP activity would be noticed. This may explain the early
lag phase observed. Similar changes could happen to higher polymers. Upon further incubation, degradation to small peptide(s) with no AFP activity starts to occur. Whether the degradation follows polymerization or occurs from monomer is not yet clear. Nevertheless, the sequence of changes from monomer to polymer to degradation products may explain the rapidly accelerating rate observed later in the incubation (Figures 1, 2).

No AFP activity was detected in AFP treated with sulfhydryl reagent. In the presence of diithioerythritol, the polyacrylamide gel pattern suggested (Figure 8C) that AFP was completely degraded into smaller molecules. Perhaps these low-Mr degradation products do not produce a peak by IED because they do not form a complex with anti-AFP during electrophoresis. Even if antigen–antibody binding is present, the much smaller size of the degradation products may prevent the formation of a precipitin line. However, in the RIA procedure, a competing-binding assay, molecules of smaller size might show activity as long as the peptide contains the antigenic determinant(s). It is not clear why AFP was more rapidly inactivated in the presence of the proteolytic enzyme inhibitor. Study of the effect of other types of inhibitors may clarify these findings. However, our experiments involving the sulfhydryl reagent suggest the presence of trace amounts of proteolytic enzyme in the AFP preparations. AFP, like albumin, contains many intramolecular disulfide linkages (2); it therefore seems reasonable to suppose that the AFP molecule unfolds upon treatment with sulfhydryl reagent, becoming more accessible to proteolytic enzymes. If so, a better method is needed for removing or inhibiting the proteolytic enzyme. The extent of contamination probably varies with each preparation.

The presence of polymers or degradation products, or both, in AFP preparations undoubtedly affects the quality of immunological reagents used for measuring AFP. They may also affect anti-AFP preparations. Therefore, precautions must be taken during storage, isolation, purification, and experimentation to avoid the generation of polymers and (or) degradation products. We recommend the following:

- Polyacrylamide gel electrophoresis should be used to detect the presence of polymers, degradation products, or both, in AFP preparations.
- Chromatography on G-200 Sephadex gel should be used to remove polymers and degradation products.
- For long-term storage, AFP should be lyophilized and stored desiccated below 0°C.
- AFP concentrations should be maintained above 100 mg/L at all times during experimentation and during lyophilization. When sample dilution is required, phosphate-buffered saline containing at least 10 mL of human serum per liter should be used.
References


