providing a means for determination of this factor in plasma under routine clinical laboratory conditions (1). The method is based on the continuous measurement of the ammonia released from a glutamine residue of β-casein upon primary amine incorporation by the catalytic action of FXIIIa formed by thrombin activation of its zymogen FXIII. Ammonia is measured in this coupled assay of measuring the change in absorbance at 340 nm from the NADPH-to-NADP+ conversion in the glutamate dehydrogenase-catalyzed production of glutamate from ammonia and β-ketoglutarate. β-Casein was chosen as the glutamine substrate for FXIII because it reportedly provides in macromolecular form the glutamine residue most sensitive to the action of FXIIIa (2). For β-casein to be used in this photometric assay, it was necessary to dephosphorylate and acetylate the molecule (1), because these modifications prevent Ca2+-induced noncovalent associations and enzyme-catalyzed cross linking, respectively, either or both of which may cause precipitation during the assay. Other drawbacks to the use of β-casein for clinical assay of FXIII include the lack of genetic homogeneity in β-casein, which creates batch-to-batch variations that may render this protein unacceptable as a standardization substrate, and a requirement for high concentrations (20 to 30 g/L), to achieve a sufficiently high change in absorbance (ΔA) for measurement of low concentrations of FXIII in plasma and small changes in FXIIIa activity, e.g., in certain cases of FXIII deficiency and at low concentrations of FXIIIa inhibitors, respectively.

In a recent study directed toward mapping the active sites of transglutaminases, several polypeptides containing glutamine were synthesized and tested as substrates (2–4). The primary structures of these peptides were modeled after the principal amine incorporation site in β-casein. They were evaluated as substrates by measurement of radiolabeled amine incorporation.

We have now tested several of these synthetic peptides in the kinetic assay as glutamine substrates replacing β-casein (Table 1) and find that two of these (Peptides 2 and 5) provide special advantages over the protein substrate. It is apparent from the results given in Table 1 that the catalytic constants (kcat values) for these two peptides significantly exceed that for the derivatized β-casein. Consequently, they offer a pronounced improvement in sensitivity. This can be seen clearly in the four-to-sevenfold increased ΔA/min readings and the thereby higher FXIII activity, U/L of citrated plasma.

We thus recommend the use of synthetic peptides in the photometric kinetic assay for human coagulation FXIII, primarily because the enhanced sensitivity allows measurements to be made at ambient temperature and without the use of very high peptide concentrations, i.e., at less than saturating concentrations of glutamine peptide substrates. Peptides 2 and 5 (Table 1) have excellent solubility properties and therefore need no derivatization for use in this assay. In addition, the units of FXIII activity provided by their use may be universally accepted as standards because these synthetic peptides are free of the types of structural variations inherent in β-casein. Finally, the method described for preparation of these peptides (2–4), the solid-phase synthesis procedure, offers a generally reliable and convenient means for obtaining these materials, even in nonchemically oriented laboratories.

References


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Further Observations on
Presaining of Human Serum with Remazol Brilliant Blue

To the Editor:

Earlier we reported preaining of human serum with Remazol Brilliant Blue and separation of the component
proteins by conventional disc electrophoresis (1). Subsequently we suggested a couple of technical modifications to eliminate staining artefacts (diffusion and trailing of dye), thereby improving the quality of resolution (2). As a corollary to the prestaining, we introduced a novel method of raising monospecific antibodies using the immunoreactive dye–protein complex (3).

Prestaining has been an ongoing procedure in our laboratory. We now communicate some further observations based on more than 3000 serum separations analyzed after the last published report. We also discuss a third and final technical modification with the object of conserving the dye, knowing that this is at the cost of a sequential refinement of the procedure. According to the latest version, we mix serum and the soluble dye (25 mg/mL in Tris–glycine buffer) in equal proportion (1:1 by vol), warm the conjugate at 40 °C for 2 h, apply a 30-μL sample and analyze it by disc electrophoresis (vide infra). Economy of the reactive dye and elegant, reproducible separations are the specific advantages. The conventional design of Davis makes use of sample (top), spacer (middle), and separator (lower) gels, cast in hollow glass cylinders 63 mm in length (4). A major pitfall of the short separator gels (35–40 mm) is the restricted migration (25–30 mm), with crowding of the bands. If we strictly adhere to the above system, the closely set prestained bands may fuse together and present as a composite antigen, which, in turn, would raise an impure antibody. In view of this distinct limitation, we have made some changes in the classical pattern to suit our purpose. We use a 70-mm-long, 70 g/L separator gel, cast in 100-mm-long glass cylinders; we delete the sample gel to shorten the electrophoretic run; and we substitute ammonium persulfate for the riboflavin conventionally used for activation of the spacer gel. Electrophoresis is at a low current, 2 mA per gel. With this revised method the bands are not crowded and migration up to 60 mm is feasible with no fading of the color.

Yet another problem in prestaining the human serum is variation of the protein profiles because of haptoglobin polymorphism (5, 6). To probe further into this facet, we selected 100 normal sera with known haptoglobin phenotypes (Hp 2-2, 68; 2-1, 28; 1-1, 3; 0, 1), and 10 from cases of severe hepatic dysfunction. Prestaining and subsequent separation was done according to the improved version. We noticed a paucity of the prestained bands at the cathodic zone of the two haptoglobin phenotypes (1-1, 0). The pathological samples also had a similar pattern, indicative of an acquired haptoglobin deficiency. With other phenotypes the number of prestained bands varied from four to 12. The concept of negative (ineffective) prestaining is important in the analysis of human sera.

Prestaining has an immense potential in the production of high-titer monospecific antisera. A pure and potent antigen is nevertheless necessary for this purpose (3). The quality of separation is critical to antigen purity. In case of contamination a re-run of the elute would dissociate the main band adequately well from the other trace components. To procure a potent antigen we now recommend that 100 normal sera initially be prestained and separated by the improved method. Gel segments harboring the desired protein are pooled and kept immersed in 4 mL of physiological saline at 4 °C for 16 h to ensure an optimum elution of the protein. The immunization protocol and method of assessing purity of the antibody is then done according to our earlier report (3). The new method, used with several rabbits, did produce a consistently stronger antiserum against transferrin than the previous method (3).

References


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