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IMMUNOLOGICAL MONITORING OF FENTON FRAGMENTATION OF FIBRINOGEN

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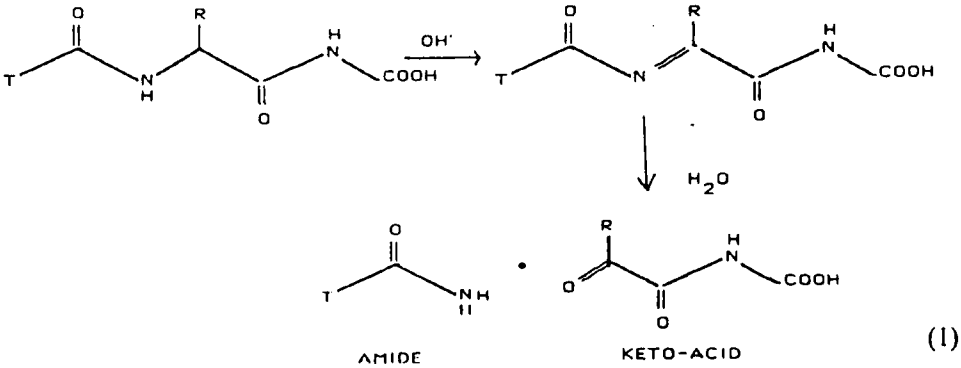
Fibrinogen is transformed into insoluble "neofibe" by reaction with up to 100 μM Cu(II) and 1.5 mM ascorbate. The soluble peptides which are released during the reaction can be monitored by amino acid analysis and by measuring released keto-carbonyl (with DNPH). Immunologic characterization of the soluble peptides, with anibodies directed against fibrino-peptide A (FPA) clearly show the release of this epitope, optimally at 50 μM Cu(II). Anti-FPB gives no evidence for the release of that epitope. However, N-terminal amino acid analyses reveals the presence of 3 peptides terminating in ALA (alpha chain FPA), GLU (beta chain FPB) and SER/ASP (unknown). The release of fibrinopeptides is interpreted within the context of a general mechanism for OH'-induced peptide chain cleavage via intermediate Schiff-base hydrolysis.

KEY WORDS: Hydroxyl radical, Fenton reaction, Fibrinopeptide release, peptide cleavage, ascorbate, fibrinogen.

INTRODUCTION

The general mechanism by which free radicals induce peptide chain cleavage involves the conversion of an amino linkage into an unstable Schiff base, the hydrolysis of which leads to a chain break. [1-4] The amino terminal fragment expresses an amide carboxy terminus, and the carboxy fragment terminates with an alpha keto groups (illustrated in Eq. 1).



(1)

Thus, fibrinogen exposed to a site-specific Fenton reaction exhibits cleaved peptide bonds at relatively specific regions.[5-7] SDS-PAGE evidence indicated that free radicals induced site-specific cleavage of the three (α, β & γ) constituent proteins chains of fibrinogen. There also appeared to be evidence for the release of relatively small

peptides (MW < 2000 daltons) which tested positive with dinitrophenyl hydrazine (DNPH).[7]

Based on these results, it was decided to further explore this coagulation pathway with immunological probes.

MATERIAL AND METHODS

Human fibrinogen from Kabi AG, Grade L (Stockholm, Sweden), ascorbate, copper sulfate, Tris, and other analytic grade reagents from Sigma (St. Louis, MO). Reagents and salts were diluted with, and fibrinogen dialyzed against 0.015 M Tris, 0.15 M NaCl, pH 7.4 buffer. Fibrinogen concentrations were determined by measuring the Abs_{280} and using a conversion factor of $E^{1\%}_{280} = 15$.

Typically, 200 ml fibrinogen (1 mg/ml final) was mixed with 10 μl stock Cu(II), mixed for 3 min, and ascorbate added. After 1 hr, the reaction mixture was vortexed and solid neofibe centrifuged down. The supernatant was decanted, heated at 65°C to heat denature any remaining fibrinogen, and further centrifuged. This supernatent was tested for fibrinopeptide A (FPA) by ELISA technique, utilizing antibody from a commercial FPA kit (Stago, France) using peroxidase conjugated goat-anti-rabbit IgG. Abs. 492 of the wells were measured in an ELISA plate reader (Molecular Devices). Controls consisting of the supernatants from heat denatured fibrinogen,

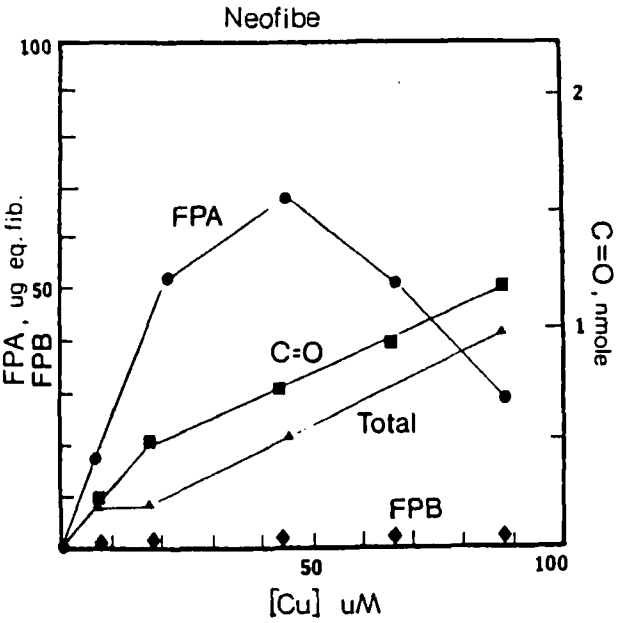


FIGURE 1 The soluble peptides (fibrinopeptides FPA or FPB, equivalent to μg/ml fibrinogen), versus Cu (II) concentration with 1.5 mM ascorbate. The peptides are determined by ELISA utilizing antibodies directed against synthetic FPA/FPB. For comparison, the supernatant carbonyl, and the relative % total fibrinogen converted into neofibe (% Total), are also shown as functions of the Cu (II) levels in the reaction mixture.

from thrombin-induced fibrin and reptilase-induced fibrin B (retaining fibrinopeptide B) were also employed.

A similar assay for fibrinopeptide B (FPB) was carried out, utilizing a polyclonal antibody to FPB produced by intramuscular injection of rabbits with FPB-albumin (bovine) incomplete Freund's adjuvant, with booster shots every 3 weeks. After 2 months, blood was withdrawn and the resulting serum tested by ELISA against pure FPB standard.

Amino acid analysis of the supernatant hydrolyzed in 6 N HCl (110 °C, 24 hr) was carried out on an LKB Amino Acid Analyzer. N-terminal amino acid analysis of the supernatant was carried out on a Bio Systems Analyzer coupled to a amino acid calibrated Spectra Physics HPLC system.

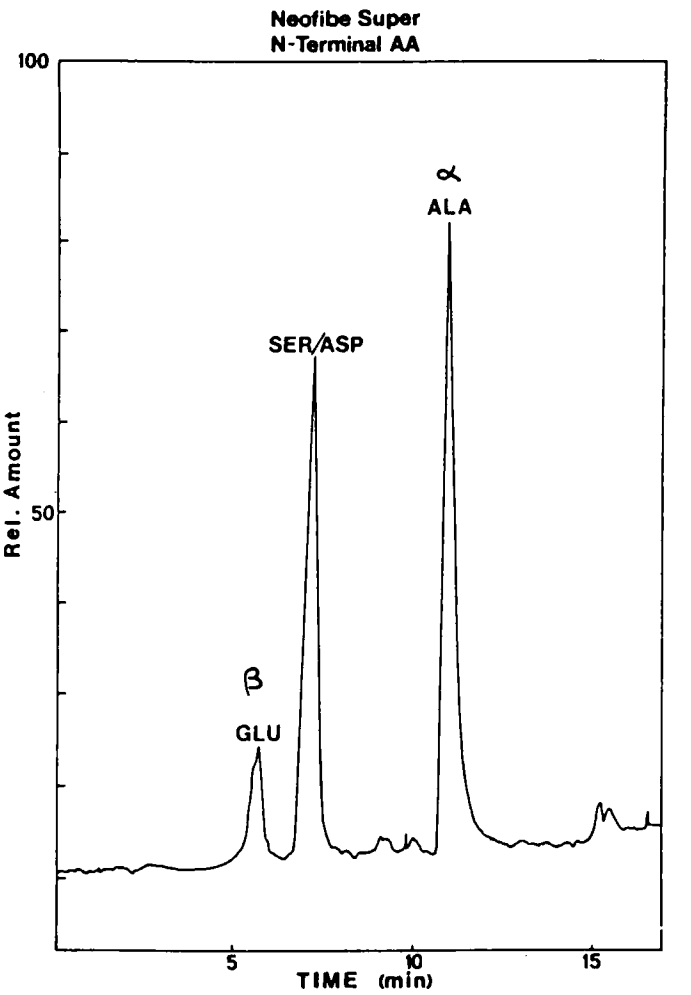


FIGURE 2 N-terminal amino acid of peptides in neofibe supernatant.

RESULTS AND DISCUSSION

ELISA immunoassays of the supernatant indicate that a peptide containing the FPA-epitope is released as a consequence of the Fenton reaction (Figure 1). This is confirmed by N-terminal amino acid analysis, which reveals the presence of three types of peptides terminating with alanine, glutamate and serine/asparagine (Figure 2). As alanine and glutamate represent the N-termini of the alpha and beta proteins respectively, these results indicate that exposure of fibrinogen to Fenton reaction conditions releases peptide analogues of FPA and FPB, and an uncharacterized fragment (neopeptides *A*, *B*, *X* tagged as NP*A*, NP*B* and NP*X* respectively). The as-yet uncharacterized features of NP*A* and NP*B* include the identity of their carboxy termini, and the amino acid side chain which has been converted to keto-positive moiety. These fragments probably were released by a single chain cleavage reaction. (Eq. 1). However, none of the native fibrinogen protein chains terminates in serine or asparagine, suggesting that NP*X* is probably derived from a double cleavage reaction.

These experiments demonstrate that release of fibrino-peptides analogues of FPA and FPB via a free-radical mechanism indicates that fibrinogen activation is not an exclusive feature of the classic coagulation cascade through thrombin, but may occur through a site-specific Fenton reaction.

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