Induction of proteolytic activity in serum by treatment with anionic detergents and organic solvents[1]

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Summary. Using casein plates as a sensitive assay for proteolytic activity, it was observed that sodium-dodecyl sulfate (SDS) and other anionic detergents induce caseinolysis when mixed with sera and plasma. Caseinolysis was dependent on the presence of plasminogen in the fluids and could be blocked by inhibitors of serine proteases and antibody to plasminogen. Similarly, organic solvents such as isopropanol induced caseinolysis after mixing with plasma, but not normal serum. Isopropanol dissociated complexes of a_1-antitrypsin or a_2-macroglobulin with trypsin preformed in vitro. As both SDS and organic solvents are widely used in biochemical investigations of biological fluids, attention should be paid to the possible induction of proteolysis.

During studies investigating the presence of proteolytic enzymes in biological fluids using casein plates as substrate, we observed caseinolysis when the fluids were mixed with anionic detergents such as sodium-dodecyl sulfate (SDS) and dextran sulfate (DS), or with organic solvents such as isopropanol, whereas there was no caseinolysis when fluids were mixed with buffer. We therefore investigated the mechanisms by which these substances induce caseinolysis. Caseinolysis due to anionic surfactants depends on plasminogen activation whereas isopropanol or methanol act by dissociating proteinase-inhibitor complexes.

Materials and methods. Agarose was purchased from Hoechst-Behring AG (Frankfurt, FRG), rabbit antiserum against human plasminogen, and normal rabbit serum from Dako (Copenhagen, Denmark), Trasylol® from Bayer (Leverkusen, FRG), suramin from Rhône-Poulenc (France), urokinase from Calbiochem (Lucerne, Switzerland) and dextran sulfate (mol.wt 500,000) from Pharmacia (Uppsala, Sweden). The other reagents were from Merck (Darmstadt, FRG). Plasma (from heparinized blood) and sera used in these experiments came from samples sent to the laboratory for rheumatoid factor determinations or were obtained from healthy persons.

Casein plates were prepared in polystyrol Petri dishes (Nunc (Roskilde, Denmark)), under sterile conditions, by a method similar to that of Bjerrum et al.[3]. Casein was dissolved in 0.1 M NaOH (2%, w/v) and titrated to pH 8.0 with 0.1 M HCl. Water was added to yield a 1% clear solution in about 0.1 M NaCl. This solution was sterilized by filtration through a 0.45 µm pore size membrane, and heated to 56 °C in a thermostatic water bath. An agarose suspension (2%, w/v) in 0.1 M Tris-HCl buffer, pH 8.0 containing 20 mM $CaCl_2$ and 0.04% (w/v) NaN_3 was dissolved in a boiling water bath, sterilized in an autoclave at 120 °C for 15 min, and then cooled down to 56 °C in a thermostatic water bath. The agarose and casein solutions were mixed thoroughly (1:1) and the resulting milky solution was poured into sterile polystyrene Petri dishes (5 cm diameter, 3.9 ml/dish), yielding homogenous 0.5% casein, 1% agarose-plates which were stored at 4 °C before use.

Samples were diluted with an equal volume of the activator or phosphate buffered saline (PBS), incubated for 1 h at room temperature, and then applied (5 µl) into punched out wells (2 mm diameter) in the casein-agarose plates. In some experiments proteinase inhibitors were added to the samples and the activators or put into adjacent wells in order to observe double diffusion pictures. The plates were kept in a moist chamber at room temperature. Digestion of casein was read every 24 h for up to 12 days as the surface of the lysed area and the volume of casein lysed calculated after correction for the volume of the well. The activity was expressed in µg of casein lysed in 24 h. 10^{-15} moles trypsin still lysed 20 µg of casein in 24 h.

a_2-Macroglobulin was purified from freshly drawn human blood and a_2M-trypsin complexes prepared as described elsewhere[4]. a_1-Antitrypsin was prepared from out-dated human plasma according to Pannel et al.[5]. Rabbit γ-globulins, either from normal serum or antiserum against human plasminogen, were purified by ion-exchange chromatography on DEAE-cellulose as described by Fahey and

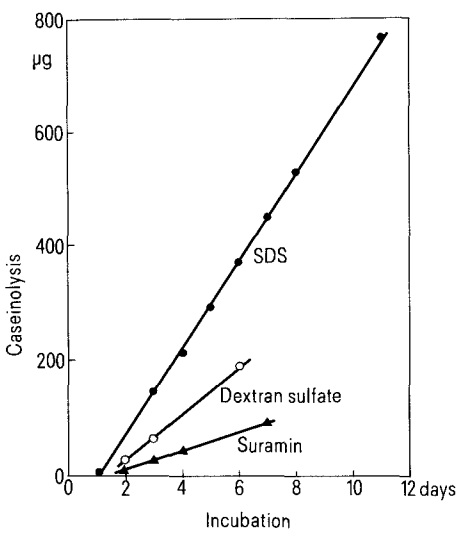

Figure 1. Time course of digestion of casein by plasma activated with sodium-dodecyl sulfate (SDS) (0.3%), dextran sulfate (64 mg/ml) and suramin (14 mg/ml).

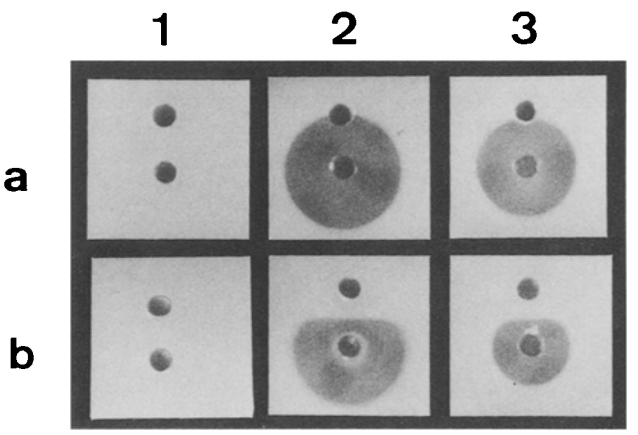

Figure 2. Casein-agarose plates, read after 48 h incubation. Caseinolysis is visible as dark discs. The lower wells were filled with normal human plasma mixed with buffer in 1a, b, with urokinase (10 plough units) in 2a, b and with sodium-dodecyl sulfate (0.3%) in 3a, b. The upper wells in the first row (a) were filled with normal rabbit IgG; the upper wells in the second row (b) were filled with IgG of an antibody against plasminogen. The distortion of the pattern of lysis indicates inhibition.

Terry[6]. Plasminogen was removed from human plasma by chromatography on lysine-sepharose according to Radcliffe and Heinze[7].

Results. Effect of anionic detergents. When plasma or sera obtained from healthy persons or patients with rheumatic complaints were mixed with SDS, DS or suramin and put into the wells of the casein plates and incubated at room temperature, caseinolysis, after a lag period of 1-2 days, proceeded in a linear fashion (fig. 1). When trypsin was used in the casein plates, no such lag period was seen. Plasma or sera mixed with PBS alone gave no lysis. Plasma or sera were best activated when 0.3% SDS was added; both higher and lower concentrations were less efficient. With DS the optimal dose was 50 mg/ml and with suramin 14 mg/ml. Soybean trypsin inhibitor (1 mg/ml), Trasylol® (1000 U/ml) or tosyl-L-lysine chloromethylketone (50 µg/ml) added to the samples inhibited caseinolysis. This suggests that a serine protease was activated. When rabbit IgG prepared from an antibody against plasminogen was added in wells adjacent to wells with SDS-activated plasma, proteinolysis was inhibited analogous to the diffusion pattern. IgG from normal rabbit serum had no effect (fig. 2). We therefore passed normal human plasma through a lysine-sepharose column to remove plasminogen[7]. Such plasma could no longer be activated by SDS or by urokinase. When the eluted fraction containing plasminogen was added back, activation by both agents occurred normally, whereas no activity was detected after activation with PBS. The eluted plasminogen, when put directly into casein plates, had a slight spontaneous proteolytic activity. It could be further activated by SDS.

Effect of organic solvents. Isopropanol mixed with serum (37.5%) induced similar proteolysis in casein plates as SDS. However, isopropanol had no effect with plasma, suggesting that it might act upon a protease generated during coagulation and subsequently inhibited by plasma inhibitors. When isopropanol was mixed with preformed α_2-M-trypsin complexes (5 µg trypsin, molecular ratio 1:2) or α_1--anti-proteinase-trypsin complexes (5 µg trypsin, molecular ratio 1:1), caseinolytic activity was induced, suggesting dissociation of protease inhibitor complexes. The other organic solvents tested (methanol, ethanol, dimethyl formamide and dimethysulfoxide) gave similar results.

Discussion. When sera were mixed with either anionic detergents or organic solvents, proteolytic activity was induced through different mechanisms. With SDS, DS and suramine, negatively charged detergents, it is probable that plasmin was induced to digest casein. This was shown with the experiments with the protease inhibitors, with the antibody to plasminogen and the removal of plasminogen from the plasma samples. In addition, purified plasminogen could be induced by SDS to digest casein. In vitro, plasminogen is activated by various plasminogen activators from plasma and tissues, kallikrein and factor XII through limited proteolysis, or by alteration of conformation[8-12]. DS has been shown to activate factor XII and prekallikrein[13], to decrease the action of α_2-plasmin inhibitor[14], to activate complement enzymes[15,16] and to enhance activation of plasminogen by urokinase[17]; the anionic detergents used in our experiments could activate plasminogen by any one of these mechanisms. A direct effect on plasminogen or plasminogen activator from plasma which are separated and eluted together on lysine-sepharose columns[7], seems most probable. We did not, however, establish the mechanisms of activation.

These results are of interest for 2 reasons. First, SDS treatment is used before acrylamide electrophoresis to determine molecular weight. Heating to 100 °C as suggested by Weber et al.[18] is not always done. The proteolytic activity often seen with partially purified biological materials[18] might partially be due to activation of proteases. Secondly, some polyanions such as DS and suramine are unspecific activators of B-lymphocytes, at least in culture[19,20]. As proteases have a similar effect on B-lymphocytes[21-23], DS and suramine might act through protease activation at the cell surface. Involvement of proteolytic steps during B-lymphocyte activation have been proposed[24,25].

Caseinolysis induced in sera by organic solvents is different. No effect was seen on plasma. Thus it is unlikely that the solvents used during our experiments acted, like chloroform, on factor XII and cofactors[8,26], which are all present in plasma. Both complexes of α_2-macroglobulin and α_1-antiprotease preformed in vitro with trypsin could be induced to digest casein. The most probable mode of action is denaturation of these inhibitors by the solvents, as has already been discussed[27]. Alcohol precipitation is widely used for fractionation of serum proteins and lipoproteins. We found direct caseinolytic activity in commercially available Cohn fractions III and IV (unpublished results). Such proteolytic activity in serum fractions might modify results of further purification.

In the present experiments, we have demonstrated that proteolytic activity can be induced in biological fluids after mixture with detergents and solvents often used in biochemical research. Appropriate controls are mandatory in experiments using SDS and organic solvents to assess modification of the results by induction of proteolytic activity.

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Changes in protein synthesis in heat-treated and normally germinating conidia of *Neurospora crassa*

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Summary. The rate of protein synthesis, as measured by pulse-labeling with [^{3}H] leucine, and the level of polysomes were found to be reduced considerably in conidia of *Neurospora crassa* growing isometrically at 46 °C, when compared to those in conidia normally germinating at 25 °C.

Incubation of conidia of *Neurospora crassa* in a nutritional medium with 2% sucrose at 46 °C for 15 h prevents formation of their germ tubes and induces an isometric enlargement of spores, which is approximately twice the normal 'swelling' at 25 °C[2,3]. Protein synthesis is required for germ tube outgrowth from fungal spores[4]. It was therefore of interest to compare levels of protein synthesis and polysome formation in normally germinating conidia at 25 °C with those occurring in heat-treated, overswollen conidia.

Material and methods. Wild-type *Neurospora crassa,* strain Lindegren 354 A, was grown in Fernbach flasks containing solid nitrate minimal medium for 2 days in the dark at 25 °C and 4 days under constant aeration with humidified air in the light. Conidia were harvested with sterile distilled water, filtered to remove mycelium and washed by centrifugation. They were inoculated at a concentration of 5×10^6 per ml in 500 ml conical flasks containing 150 ml Vogel's medium[5] with 2% sucrose. Flasks were incubated in a water-bath for 15 h at 46 °C under reciprocal agitation. Control cultures were grown at 25 °C under the same conditions[3].

Total cellular protein was determined on aliquots after hydrolysis in 1 M NaOH by the method of Lowry et al.[6] using bovine serum albumin as standard. Dry weight measurements were made by filtering samples on preweighed filter papers, washing with acetone and drying to constant weight in a desiccator. For the measurements of the rate of protein synthesis, aliquots of 5 ml cultures were removed at intervals and incubated in a shaken water-bath for 15 min with 0.5 µCi/ml of L-[4,5-^{3}H] leucine (Radiochemical Centre, Amersham, England; sp. act. 53 Ci/mmole) at appropriate temperatures. 2 ml of 10% trichloroacetic acid was added to duplicate samples of 2 ml each, placed in a water-bath at 100 °C for 15 min and chilled. Suspensions were filtered on Whatman GF/C filters, washed with ice-cold 5% trichloroacetic acid, then with alcohol, dried and counted in a toluene-based scintillation solution in a Nuclear Chicago scintillation spectrometer. The linearity of incorporation was verified by analyzing samples at 5 and 10 min pulses.

Isolation of polysomes was made as described previously, at 4 °C using sterile glassware and solutions[7]. After harvesting cultures with cycloheximide (50 µg/ml) to prevent ribosomal run off[8], the conidia were broken for 180 sec on a Vortex mixer with glass beads and a high ionic strength homogenization buffer (pH 8.5, 50 mM Tris-HCl, 400 mM KCl, 50 mM Mg acetate, 200 mM sucrose). After centrifugation at 16,000×g for 15 min, the supernatant was layered on to a pad of 1 M sucrose in buffer B (pH 7.8, 50 mM Tris-HCl, 200 mM KCl, 10 mM Mg acetate) and centrifuged at 160,000×g for 4 h. The ribosomal pellet was resuspended in buffer B and centrifuged for 40 min at 190,000×g on a 10–40% (w/v) linear sucrose gradient made up in buffer B. Gradients were scanned at 260 nm and the areas under the polysome and monosome peaks were calculated to determine the percentage of ribosomes in polysomes. All results are representative of several experiments.

Results and discussion. During isometric growth of the conidia of *N. crassa,* owing to the elevated temperature of incubation (46 °C), the amount of total proteins and dry

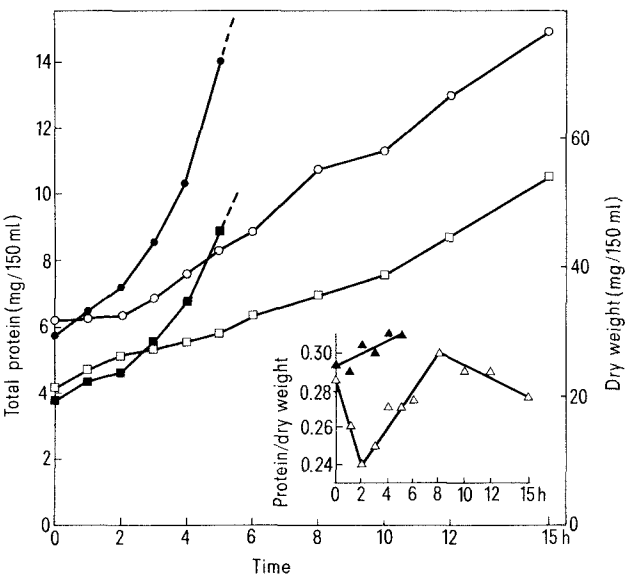

Figure 1. Changes in protein content and dry weight during the 1st h of germination at 25 °C and heat-treatment at 46 °C in shaken cultures of *N. crassa* in Vogel's medium at a concentration of 5×10^6 conidia/ml. Protein content of germinating conidia (●) and heat-treated conidia (○), and dry weight of germinating conidia (■) and heat-treated conidia (□). The inset shows the ratio protein (mg) per dry weight (mg) during germination (▲) and heat-treatment (△).