Gel Diffusion Methods for Determination of Protease and Protease-Inhibiting Activities

In preliminary studies on protease activities and in studies on the effect of inhibitors or activators on proteases, rapid answers are often needed on three questions. One is the pH optimum of the enzyme, the second is the localization of inhibitors (or activators) to particular protein fractions, e.g. in an electrophoretic pattern, and the third question is the specificity of the enzyme activity to various substrates and the effects of various ions.

In the present report we describe two gel diffusion methods for these purposes, one of them in combination with electrophoresis. Special attention is drawn to the trypsin-inhibiting activity in porcine colostrum and in serum and urine of the newborn piglet in connection with the onset of colostrum ingestion.

Radial diffusion assay. A 3% solution of agarose (l'Industrie Biologique Française) was melted at 100°C, cooled to 45°C and mixed with solutions of casein (BDH), serum albumin (porcine fraction V, NBC) or bovine haemoglobin (Sigma), giving a final agarose concentration of 2.25% and a substrate concentration of 0.1%. The agarose-substrate solution was pipetted onto microscope slides (3.0 ml on each). In the solidified gel, wells of various shapes and arrangements were then punched (Figure 1). For the determination of the optimum pH of the enzyme or for studying the effects of various ions and ionic strengths, the solution in the solidified gel could be exchanged by immersing the slides for 30 min in a cuvette containing the solution to be studied.

The wells were filled with samples (10 µl) to be analyzed for protease or protease-inhibiting activity. After incubating the slides at 37°C for 3–4 h, they were immersed in 2% acetic acid for 1 h. The enzymic reaction was thus stopped and the undigested substrate precipitated. The agarose layer was dried to a thin film and stained with amido black. Proteolytic activity was indicated by circular non-stained zones around the wells (Figure 1). When a substance with inhibiting activity was placed in a neighbouring well a reduction in the reaction zone was obtained (Figure 1).

The area of the zone of reaction is proportional to the enzyme activity. This was demonstrated by the use of samples of known concentrations of trypsin, chymotrypsin and pepsin (Sigma). This principle has earlier been demonstrated for pepsin activity on haemoglobin by SAMLOFF et al.[1], for DNase on DNA by JARVIS and LAWRENCE[2] and for protease inhibitors in some animal and plant materials by FOSSUM[3]. The reduction of the zone of reaction by the presence of an inhibitor in a neighbouring well or trough (Figure 1) is approximately proportional to the inhibiting activity. This was controlled by serial dilutions of soybean trypsin inhibitor (Sigma) and other samples containing inhibitor. The inhibiting activity was controlled by the spectrophotometrical method of FRITZ et al.[4] using BAPNA as a substrate (Sigma).

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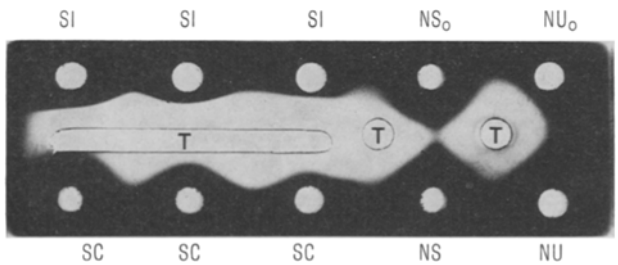

Fig. 1. Trypsin and trypsin inhibiting activity in a radial diffusion test in agarose-casein gel (photo). The wells in the middle row contain trypsin, T (1 mg/ml). The wells in the upper row contain soybean inhibitor, SI (1.0 — 0.1 — 0.01 mg/ml), serum, NS_0, and urine, NU_0, from a new-born unsuckled piglet. The wells in the lower row contain porcine colostrum, SC (undiluted — 1:10 — 1:100), serum from a piglet, suckled for 3 h, NS, and urine from a piglet having suckled for 14 h after birth, NU. Incubation at 37°C for 3.3 h. Black areas indicate undigested casein.

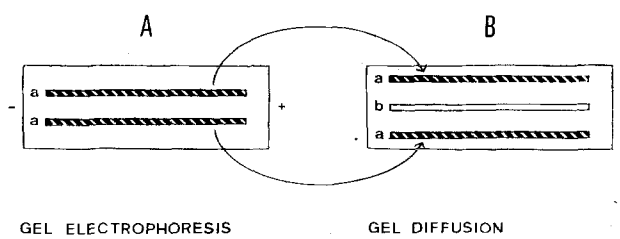

Fig. 2. Gel electrophoresis in combination with gel diffusion. The electrophorized material (A,a) was cut out and placed on another gel (B) containing agarose-casein. The trough (B,b) contains trypsin.

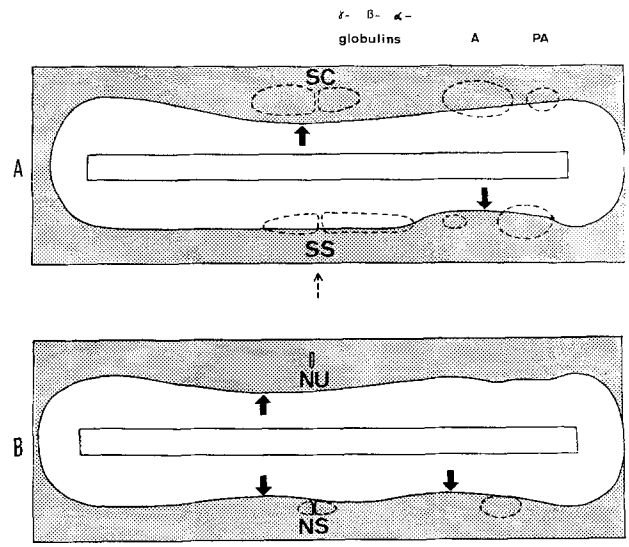

Fig. 3. Gel electrophoresis in combination with gel diffusion. A) Inhibiting zones (arrows) are noted in the γ-globulin region for porcine colostrum (SC) and in the albumin-α-globulin regions for porcine serum (SS). B) Inhibition is found in the γ-globulin zone for urine (NU) from a piglet having suckled for 14 h. In the serum of a piglet (NS) having suckled for 3–4 h after birth inhibition was noted in both the γ-globulin and in the albumin-α-globulin zones. The dotted area represents undigested casein. The zones included by dashed lines are the electrophoretically separated protein fractions. Dotted arrow indicates site of application at electrophoresis.

Our experiments demonstrate that the use of casein as a substrate, as opposed to serum albumin and haemoglobin, results in sharper borders between the zone of reaction and the area containing the undigested substrate. An advantage of the method of radial diffusion described above is that casein can be utilized as a substrate in studies on enzymes with any pH-optima. To illustrate this, if the agarose casein mixture is prepared at neutral or basic pH values and then the slides are immersed in buffer of low pH (e.g. 1 or 2 for pepsin studies), the casein precipitates in small aggregates which are homogenously distributed within the gel. It is not possible to get a homogenous dispersion of casein in the gel if it is mixed directly with agarose and buffer below pH 4.5, since the casein is precipitated and aggregated.

Electrophoresis followed by diffusion. This method has been developed in order to provide a rapid electrophoretical localization of protease inhibitors or activators in samples from body fluids and organ extracts.

Agarose electrophoresis (Veronal-HCl buffer, pH 8.6) is first performed on the solution already proved to contain inhibitors by e.g. radial diffusion. After electrophoretic separation, longitudinal slides are cut out from the gel into which the proteins have migrated (Figure 2A), and placed upon another agarose gel (Figure 2B). This gel contains 2.25% agarose and 0.1% substrate (casein). In the latter gel, a trough is punched and the enzyme solution is poured in. The incubation is performed at 37°C for 4–5 h. Molecules diffuse from the slices into the underlying gel. The enzyme in the longitudinal well diffuses into the gel and digests the substrate. However, in those positions where inhibiting substances have diffused from the applied slice there is no, or a reduced, digestion. These results are clearly observed after the undigested casein has been precipitated with acetic acid and stained with amido black (Figure 3).

Figure 3A shows one test where a trypsin inhibitor of porcine colostrum is localized to the γ-globulin region, and another test where the tryspin-inhibiting activity of the α-globulin region in porcine serum is shown. Figure 3B is a test of serum from a new-born piglet which ingested its first meal of colostrum 3 h earlier. Note the trypsin-inhibiting action of both serum and colostrum. Figure 3B also demonstrates that urine from a 14-hour-old suckling piglet exhibits trypsin-inhibiting activity in the γ-globulin zone. This may depend on the fact that a trypsin inhibitor actually has been absorbed from colostrum via the gut epithelium to the blood and then via the kidneys to the urine[5].

This is a good example of the applicability of this qualitative test in a situation when the measurement of the total trypsin-inhibiting activity would have given incomplete physiological information.

Zusammenfassung. Es werden zwei Methoden (Diffusion und Elektrophorese in Agarose-Kasein) für quantitative und qualitative Bestimmung von Inhibitoren und Aktivatoren verschiedener Proteasen beschrieben und die Trypsininhibitoren in Kolostrum, Serum und Urin von neugeborenen Schweinen besonders untersucht.

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A Double-Labeled Frozen Section Technique for Studying Distribution of H^3-Norepinephrine[1]

The arterial wall is stratified into tunicae intima, media and adventitia with differing diffusion characteristics and catecholamine uptake capacities. Catecholamines, either released from the sympathetic nerve terminals or applied exogenously, do not distribute uniformly throughout the arterial wall. Their distribution profiles are of physiological and pharmacological consequence[2].

A frozen section technique has been devised to study the transmural distribution of norepinephrine in the rabbit aortic strip[3]. By this technique, the kinetics of entry of tritiated norepinephrine (H^3–NE) into the arterial wall has been characterized. Briefly, the arterial strip was exposed to H^3–NE before it was frozen and sectioned into thin slices parallel to the intimal surface. The tritium content of each slice was assayed and plotted against the depth of the slice within the arterial wall to obtain a distribution profile[4]. As the catecholamines are subject to cellular uptake, differentiation betwen the cellular and extracellular components in the profile is often necessary. Rinsing the tissue with saline solution can largely remove the substance from the extracellular spaces, but this will most likely mobilize some of the cellular component as well. Further, rinsing is precluded in kinetic studies in which the tissue must be instantaneously frozen at the end of exposure to H^3–NE. This problem has been met by subtracting the H^3 material expected in the extracellular space from the total H^3 content to derive cellular component in each slice. The aortic extracellular space has been independently determined using the same frozen section technique but substituting C^{14}-inulin for H^3–NE[5]. The direct application of the average extracellular space (inulin space) thus obtained to studies of distribution of H^3–NE made on separate aortas, suffers from the considerable variations between aortas and between slices from the same aorta.

The present work is an attempt to circumvent this drawback. The aortic strip was doubly labeled with H^3–NE and C^{14}-inulin. Strips of the rabbit thoracic aorta were soaked in 0.32 μg/ml of C^{14}-inulin (inulin-carboxyl-C^{14}, 3.0 mc/g, New England Nuclear, Boston, Mass.) singly, or in combination with 0.5 μM H^3–NE (1-norepinephrine-7-H^3, Amersham/Searle, Arlington Heights, Ill.). After 55 min of soaking in a 10 ml tissue bath, for an additional 5 min the strips were superfused with the same medium at a rate of 3 ml/min. At the end of superfusion, the tissues were immediately frozen, sectioned at 24 μm and

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