SPECIFICITY OF TRYPSIN ADSORPTION ONTO CELLULOSE, GLASS, AND QUARTZ[1]

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Under conditions typical of some protein purification procedures, there is extensive adsorption of certain conformers of trypsin onto dialysis membranes and glass and quartz powders. If our results are typical for many proteins, then purification methods carried out in glass or which involve dialysis could, if not carefully controlled, damage or destroy biological potency inadvertently. That is, as will be seen below, there is extensive loss of tryptic activity under conditions normally used in dialysis; or alternatively, the use of different pieces of glassware which are presumably identical but which are actually from different batches of glass could lead to large variations in the amount of a valuable biochemical preparation which is either recovered or which retains its biological activity during duplicate preparative procedures. Although we stress the potential hazards of adsorption, specificity of adsorption onto glass and resins has been utilized in purifying some enzymes (Rasmussen, 1954; Kogut et al, 1956; see review James and Augenstein, 1966).

DIALYSIS MEMBRANES The cellulose membranes (approx. 28 μ thick when wet) were obtained from Scientific Glass Apparatus Co., Bloomfield, N. J. and were washed several times with distilled water to remove glycerin and other organic materials. For a single adsorption measurement, one piece of membrane 2.5 x 2.2 cm was put for the desired time into a given protein solution at 4°C in a polystyrene vial. After removal, we determined the amount of adsorbed protein by measuring directly the optical density at 220 nm of protein and membrane against a control piece

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of membrane in a Cary 15 recording spectrophotometer. This value agreed with the decrease at 220 nm in the supernatant of the solution from which the protein was adsorbed. The amount of active enzyme adsorbed onto the membrane (and correspondingly that removed from solutions) was determined by a standard assay using benzoyl arginine ethyl ester as substrate (Worthington, 1957).

Both the rate and the amount of adsorption increase with protein concentration. However, plots of either adsorption or desorption with time can be resolved as the sum of two or at most three exponential components suggesting that different trypsin conformers adsorb and desorb at different rates. The maximum OD of 0.165 for the 5 μM solution shown in Fig. 1a corresponds to a uniform layer of protein 260 A thick on each side of the membrane -- with 72 hours adsorption in 3 μM trypsin the value increases to 1300 A.

The concentration of either salt or buffer ions as well as pH also affects greatly the amount of protein which adsorbs. The lower the ionic concentration the greater the adsorption (Fig. 1a) -- e.g., in only distilled H_2O, up to 15% of the trypsin in 3 ml of a 3 μM solution will adsorb onto the 11 cm^2 of membrane surface in 1 hour; even with 10^{-2} M phosphate buffer present, 3% will adsorb in this time and approximately 10% when the adsorption achieves a maximum after 4 days. The data in Fig. 1b illustrate the effect of varying pH while holding the phosphate buffer concentration constant at 10^{-3}M. Clearly the values of pH for maximum adsorption are not the same for trypsin ($pH_{max.}$ = 6.3) and RNase ($pH_{max.}$ = 5.3). Thus apparently the extent of adsorption is determined by the pK of groups in both the protein and the cellulose. This plus the greater trypsin adsorption at higher temperatures suggests that chemisorption is most important in the adsorption of this protein. Unequivocal interpretation of the temperature enhancement is difficult, however, since the distribution of trypsin conformers -- which have different adsorption rates -- probably changes with temperature (Augenstein et al, 1961). Considering the different molar extinction coefficients of the two proteins, it appears that the maximum number of trypsin and RNase molecules adsorbed onto cellulose are about the same.

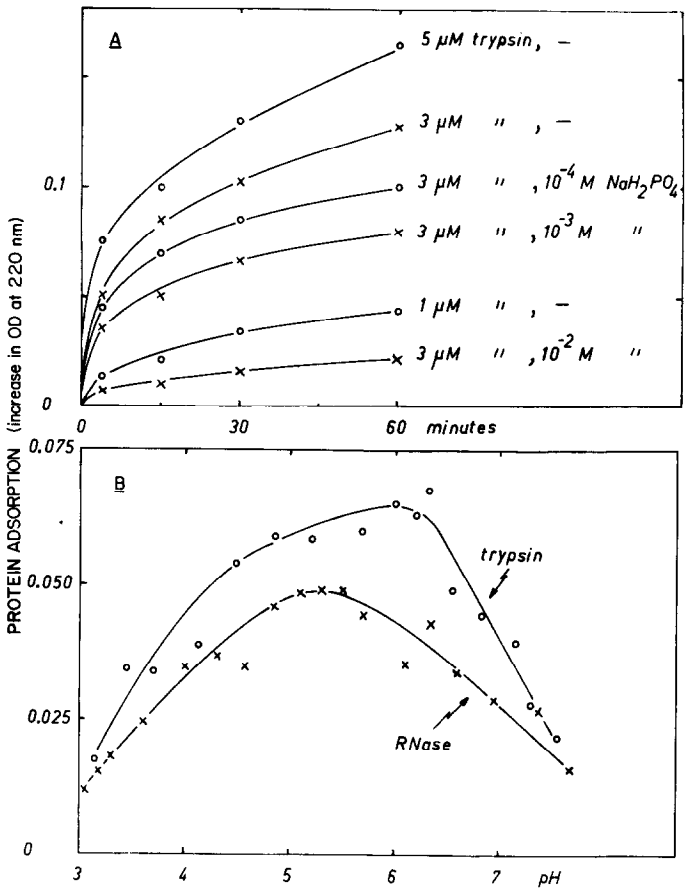

Figure 1. A. The adsorbance of trypsin onto dialysis membrane as a function of time and buffer content. B. The effect of pH on the amount of trypsin or RNase adsorbed from 3 μM solutions onto dialysis membrane in 30 minutes. The pH was maintained using 10^{-3} M phosphate buffers.

Of perhaps greatest consequence is our observation that active and inactive forms of trypsin adsorb and desorb at different rates. For example, whereas essentially no activity was lost in control solutions which contained no membrane material, total trypsin activity was lost after 48 hours in a 1 μM solution and after 72 hours in a 3 μM solution containing a piece of membrane even though 15% of the initial protein remained in the supernatant (distilled water only -- no buffer) of both solutions. Preliminary measurements of the adsorption spectra

of the trypsin molecules actually on the membranes suggest that some conformation changes occur upon adsorption. Presumably a dynamic process of adsorption, unfolding, and desorption accounts for the continual loss of activity in the supernatant.

GLASS - Soft glass spheres 13-44 μ in diameter were purchased on four separate occasions from LaPine Laboratory Supplies Co. According to the vendor, these were identical in composition and manufacture. However, as detailed below, the adsorptive behavior of these lots were far from identical, although the results were all reproducible within a given batch of glass.

Batch No. 1. Maximal adsorption was achieved when the glass beads were cleaned with concentrated HNO_3 or H_2SO_4 (less adsorbed after soaking in conc. HCl), and then washed exhaustively and dried. Presumably the rapid adsorption in twenty seconds or less (Fig. 2a) reflects the time required for thorough mixing of the powdered glass and solution. Since little activity was lost, however, this initial rapid adsorption must have involved primarily inactive material; in particular, the tryptic activity per unit mass of protein remaining in the supernatant increased appreciably following 1 minute of adsorption. The glass did not, however, remove simply by adsorption a tryptic inhibitor since the absolute amount of trypsin activity in the supernatant declined slowly with longer adsorption times indicating that active molecules were adsorbing. Further, active material was retrieved by washing the glass with buffer after adsorption. When 1 gm of glass (approximately 10^3 cm^2 of surface) was added to 20 ml of a 5 μM trypsin solution, practically all of the inactive conformers must have been removed, since addition of a second aliquot of glass to the same solution did not enhance specific activity in the supernatant and now both activity and total protein were lost at the same rate.

As with the dialysis membrane, the maximum amount of protein adsorbed per unit area of glass increased with increasing temperature. However, this was not simply adsorption of increasing amounts of trypsin molecules reversibly inactivated by thermal energy (Kunitz and Northrup, 1933). If so, a plot of -log

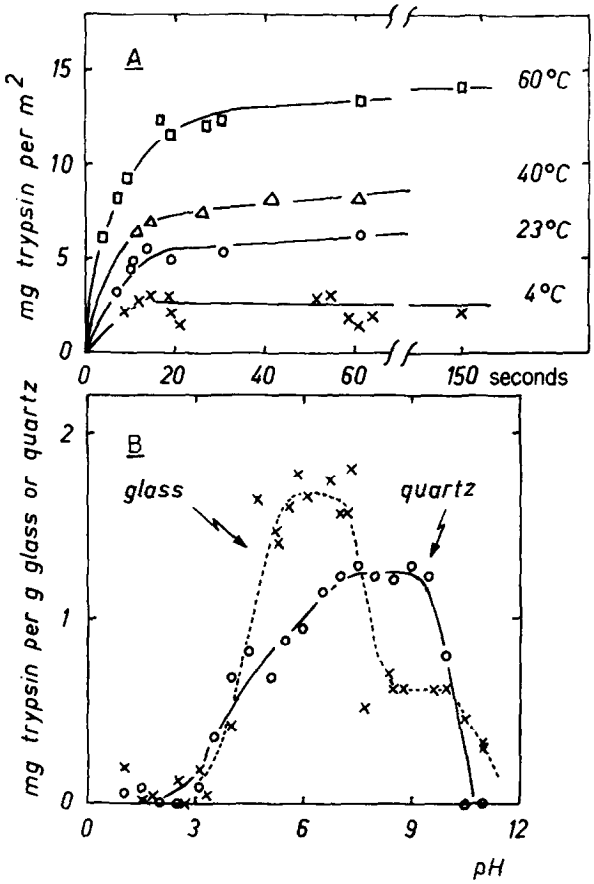

Figure 2. A **The effect of temperature on the amount of trypsin adsorbed from 20 ml of a 5 μM solution onto 1 gm of glass beads.** B **The effect of pH on the amount of trypsin adsorbed in 10 minutes onto 1 g of glass beads or quartz powders placed in 20 ml of a 9 μM solution.**

(maximum amount adsorbed) versus 1/T should have a slope corresponding to 40 kcal/mole, the value reported for reversible inactivation of trypsin (Stearn, 1949). The data in Fig. 2a are consistent with a much lower value of approximately 3 kcal/mole. At elevated temperatures, the maximum amount adsorbed is much greater than the amounts of egg albumin and bovine serum albumin which Bull (1957) found would adsorb onto powdered pyrex glass and greater than that expected for a single monolayer.

Batch No. 2. Very minimal amounts of trypsin adsorbed when concentrated

acids were used for cleaning; whereas when absolute ethyl alcohol was used, the amounts adsorbed were less than with Batch No. 1 but comparable to those reported by Bull (1957). Other differences from Batch No. 1 are: (a) Adsorption was slower and occurred in two steps -- a first plateau was reached in 1 minute and a second in about 8 minutes. (b) There was little enhancement of specific activity in the supernatant following adsorption. (c) Activity and total protein were "adsorbed" from the supernatant at almost the same rates. (d) Adsorption was slightly greater at 4°C than at 30°C. With this batch, adsorption was maximal in the pH range 4.5-7 (see Fig. 2b).

Batches No. 3 and 4. Only trivial amounts of trypsin could be adsorbed for all the methods we used for cleaning.

QUARTZ POWDERS - When quartz powders were cleaned with concentrated acids (similar to Batch No. 1 above) the amount of trypsin adsorbed was about the same as that taken up by a comparable weight of glass beads in Batch No. 2. But, since the quartz powders were of a finer grain, and thus had more surface area, adsorption per unit of area was less than that reported by Bull for pyrex or for Batches 1 or 2 above. Adsorption of trypsin onto the quartz is peaked at more alkaline pH's (7-9) than it is for the glass beads or for the dialysis membranes (Fig. 2b). Also, unlike the other two adsorbents studied, it was not possible to elute the trypsin from the quartz with phosphate buffer at pH 4.5 or 7. Similar to Batch No. 2 and contrary to Batch No. 1 of the glass, adsorption onto quartz had a slightly negative temperature coefficient.

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