THE DETERMINATION OF THE NORMALITY OF A TRYPSIN SOLUTION BY A SPECIFIC TITRATION*

Myron L. Bender, John V. Killheffer, Jr. and
Roger W. Roeske

Department of Chemistry, Northwestern University, Evanston, Illinois, and Department of Biochemistry, Indiana University, School of Medicine, Indianapolis, Indiana

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The concentration of active sites in an enzyme solution may be determined by (1) a rate assay using an (assumed pure) enzyme solution as standard or (2) a titration of active sites using a (specific) reagent which combines stoichiometrically with the enzyme active sites as standard. (Schonbaum, Zerner and Bender, 1961). The spectrophotometric titration of the active sites of α-chymotrypsin by both non-specific (Schonbaum, et.al., 1961; Hartley and Kilby, 1954; Caplow and Jencks, 1962), and specific (Kézdy, Clement and Bender, 1964) substrates has been described. This report describes a specific titrant, p-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride, for determining the absolute concentrations of trypsin solutions in an accurate and convenient manner.

The titrant which was selected because it contains the lysine backbone for which trypsin is specific, (Desnuelle, 1960) was prepared by selective removal of the t-butyloxycarbonyl group

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from p-nitrophenyl N^2-benzyloxy carbonyl-N^6-t-butyloxycarbonyl-L-lysinate (Schwyzer and Rittel, 1961; Bodanszky and du Vigneaud, 1959) by treatment with excess anhydrous HCl in ethyl acetate at room temperature for twenty minutes. After one recystallization from acetonitrile it melted at 150-152°, $[\alpha]^D_{20} = 22°$ (C = 2.2 in dimethylformamide). Analysis: Calcd. for $C_{20}H_{24}O_6N_3Cl$: C, 54.86; H, 5.52; N, 9.60. Found: C, 55.15; H, 5.74; N, 9.64. Hydrolysis of the titrant in 0.1 N sodium hydroxide produced 95-97% of the calculated amount of p-nitrophenol. Enzymatic hydrolysis at pH 6.83 gave 100 ± 0.5% of the amount of p-nitrophenol produced by non-enzymatic (alkaline) hydrolysis at the same pH, indicating the optical purity of the titrant.

An apparent instantaneous release ("burst") of p-nitrophenol followed by a slower zero-order formation of p-nitrophenol (the turnover of the enzyme) was observed in the reaction of the titrant with trypsin at pH 3.71. The effect of substrate concentration on the instantaneous "burst", π, and on the velocity, v, of the zero-order reaction is shown in Fig. 1. Eq. 1

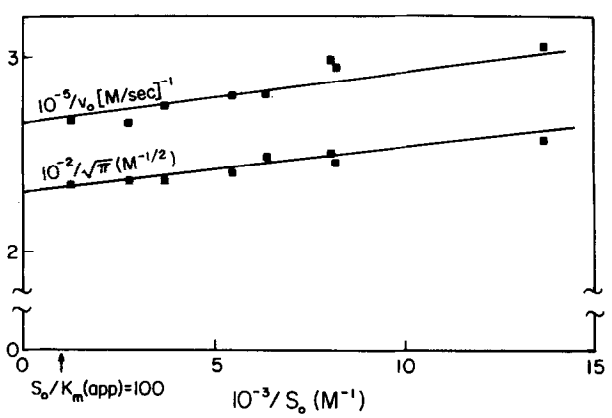

Fig. 1. The effect of substrate concentration on the titration ($1/\sqrt{\pi}$) and the turnover ($1/v_o$) of trypsin with p-nitrophenyl N^2-benzyloxy-carbonyl-L-lysinate hydrochloride.

shows the relationship between the presteady state "burst" and the enzyme concentration, (Ouellet and Stewart, 1959; Kézdy and Bender, 1962) assuming

$$\sqrt{\pi} = \frac{k_2/(k_2+k_3)}{1 + (K_m(app))/S_o} \sqrt{E_o} \qquad (1)$$

the acyl-enzyme mechanism with two rate steps, acylation, k_2 and deacylation, k_3, and assuming preequilibrium formation of the enzyme-substrate complex. Thus, if $k_2 >> k_3$ and $S_o >> K_m(app)$, the measured burst, π, will beequal to the enzyme concentration, E_o. The condition, $S_o >> K_m(app)$ may be easily met by extrapolation of the plot of $\sqrt{\pi}$ vs. $1/S_o$ to the ordinate (Fig. 1). Since the presteady state is finished before observations can be made (10 sec.), it is not possible to observe directly the rate constant k_2 and thus the ratio k_2/k_3 cannot be determined. However, previous experience with p-nitrophenyl esters (Kézdy and Bender, 1962); Zerner, Bond and Bender, 1964), indicates that k_2/k_3 ratios are usually of the order of 1000.* Thus the intercept of the plot of $1/\sqrt{\pi}$ vs. $1/S_o$ may be taken as $1/\sqrt{E_o}$. From the plot of $1/v_o$ vs. $1/S_o$, $k_{cat} = 0.199$ sec.$^{-1}$ and $K_m(app) = 1.0 \times 10^{-5}$ M. Thus, the experimental burst at our highest substrate concentration ($\sim 10^{-3}$ M) may be calculated to be 98% of the extrapolated E_o, and thus may be used for the direct titration of a trypsin solution.

For the optimal titration, a 10^{-3} M solution of the titrant was prepared in 2 vol% acetonitrile-water solution, 0.04 total formate buffer, pH 3.71, $\mu = 0.02$ (the titrant has a half-life of about 13 hours in this solution at 25°). Solutions of bovine

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* A lower limit of the k_2/k_3 ratio of at least 10 is required by the lack of observation of the presteady state after 10 sec.

trypsin (Worthington Biochemical Corp., 2x crystallized, lyophilized, TRL 6253) were prepared in the same buffer and stored at 0°. The titration was carried out at 25° by observing the formation of p-nitrophenol at 320 mμ using a Cary Model 14CM recording spectrophotometer. A procedure corresponding to method A with N-trans-cinnamoylimidazole was used (Schonbaum, Zerner and Bender, 1961), adding 3.0 ml. of the titrant solution to the spectrophotometer cuvette directly.

The equation of the burst is

$$\pi = \frac{A_2 - (1-(v_1/v_2))A_1 - A_e}{\Delta \epsilon} \qquad (2)$$

where A_2 and A_1 are the absorbancies extrapolated to time 0 from the enzymatic turnover, and from the spontaneous hydrolysis of the titrant respectively, v_1/v_2 is the ratio of volumes of the enzyme aliquot and the final volume of the reaction mixture, A_e is the absorbance of the enzyme (determined separately), and $\Delta\epsilon$ is the difference in extinction coefficients (products minus starting species) = 8171.

The enzyme molarity found in this titration is 57% of that calculated from the weight taken, assuming a molecular weight of 24,000. (Laskowski, 1955). Titrations of the same batch of trypsin by the non-specific titrants, p-nitrophenyl acetate, acetyl-DL-leucine and acetyl-DL-tryptophan p-nitrophenyl esters gave values of 57 $\pm$ 7% in reasonable agreement. Their lack of specificity precludes their use in routine titrations

The titration of a trypsin solution as described above gives precisely the same value in the presence or absence of an equimolar quantity of α-chymotrypsin, indicating the specificity of this titration. Since thrombin shows specificity toward

lysine substrates (Waugh and Buchanan, 1960) the titrant described here probably can be used for the titration of thrombin solutions and thus could not distinguish between trypsin and thrombin. The availability of a trypsin titration allows a quantitative evaluation of the kinetic parameters of this enzyme.

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