# DIFFERENTIAL TITRATION OF TRYPSIN-LIKE ENZYMES*

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In pursuing the systematic study of insect proteinases we were interested in exploring the titration of cocoonases, a new class of trypsin-like enzymes produced by silkworms (Kafatos et al., 1967 a,b). The titration of serine proteinases has been discussed by several workers (Kézdy et al., 1965; Chase and Shaw, 1967; Heidema and Kaiser, 1968; Elmore and Smyth, 1968 a,b; Tanizawa et al., 1968). The most suitable titrants are esters that react rapidly to form acyl-enzyme compounds which deacylate slowly. The simultaneous stoichiometric release of the chromophoric alcohol can then be ascribed to the formation of the acyl enzyme. For the titration of trypsin, p-nitrophenyl-p'-guanidino benzoate (NPGB) has been proposed by Chase and Shaw (1967). They observed that the reaction of NPGB with trypsin at pH 8.3 was very rapid and deacylation could not be detected. (The compound also reacted with chymotrypsin but with this enzyme deacylation occurred.) The striking similarity between trypsin and cocoonase then led us to try NPGB as a titrant for the latter. Indeed, we observed that NPGB reacted readily with cocoonase and the concommitant release of p-nitrophenol obeyed pure first order kinetics. Thus NPGB was a suitable titrant for cocoonase. It was also of interest to compare the reactivity of cocoonase and trypsin toward this rather non-specific reagent. However, when we studied the kinetics of the acylation of bovine trypsin with NPGB we found it to be complex, due to the presence of three enzymatic species with widely different reactivities. Two of these could be identified as the chromatographically distinct components of bovine trypsin described by Schroeder and Shaw (1968). By a similar analysis porcine trypsin showed the

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presence of two different enzymatic species. Gel electrophoresis could be used to demonstrate the conversion of the active enzymes to guanidinobenzoyl derivatives during titration.

## MATERIALS AND METHODS

Bovine trypsin (Lot TRL 7LA and TRL 8GA) and bovine chymotrypsin (Lot CD 16KD) were from Worthington Biochemical Corporation. Porcine trypsin, once-crystallized, was a gift from Dr. I.E. Liener. Cocoonase from Antheraea polyphemus was collected as described by Kafatos et al. (1967a). A small sample of NPGB was kindly provided by Dr. Elliot Shaw and a larger sample (Lot K-5965) was purchased from Cyclo Chemical Company. This firm also supplied 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK; Lot F-1734) and Fox Chemical Company supplied L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK; Lot F-670). SE Sephadex (C 50) was the product of Pharmacia Fine Chemicals Inc.

Chromatography of Bovine Trypsin - The SE Sephadex chromatography method of Schroeder and Shaw (1968) was followed exactly using 0.1 M Tris-HCl, pH 7.1, with 0.02 M $CaCl_2$ and 1 mmolar recrystallized benzamidine HCl (Aldrich) at 4°C. Absorption of the eluate was measured at 280 mμ and activity was measured spectrophotometrically against N-benzoyl-L-arginine ethyl ester (BAEE) at pH 8.1. The results of this chromatography were similar but not identical to those reported by Schroeder and Shaw (1968). Individual fractions were selected, combined and dialyzed against several changes of acetate buffer, pH 4, ionic strength 0.1 M at 4°C. After dialysis and determination of specific activity, the fractions were titrated with NPGB. Consistent losses in specific activity of up to 40% accompanied dialysis.

Titration Procedures - The release of p-nitrophenol was measured at 340 mμ on the Cary 15 spectrophotometer using both the normal and expanded slide wires. Stock enzyme solutions (approximately $10^{-3}$ M) were made up in 0.1 M citrate buffer, pH 3.1, and stored on ice until needed. Stock titrant solutions were $10^{-2}$ M NPGB dissolved in 4:1 acetonitrile-water.

The titrations were performed in 1.7 ml of 0.1 M sodium acetate-acetic acid buffer, pH 4.0, to which 50λ of NPGB stock solution were added. The reaction was initiated by

adding 50λ of enzyme solution, and the absorbance was recorded as a function of time. Results were corrected for the slight absorption of the enzyme at 340 mμ. Titrations of fast-reacting species or titrations at pH 8 were followed with a Durrum-Gibson stopped-flow apparatus.

Kinetic Analysis of the Trypsin Titration - The complex titration kinetics of bovine trypsin can be expressed as the sum of three independent first order reactions:

$$A_\infty - A_t = \Pi_1 e^{-k_1 t} + \Pi_2 e^{-k_2 t} + \Pi_3 e^{-k_3 t}$$

where $A_\infty$ and $A_t$ are the absorbances at time $=\infty$ and time = t respectively; $\Pi_1, \Pi_2, \Pi_3$ are the total absorbance changes produced by each trypsin species; and $k_1$, $k_2$, $k_3$, are the experimental first order rate constants of each species. The analysis made use of the fact that the rates of acylation were widely different for the three enzyme species. After 300 seconds the first two trypsins were more than 99% titrated while the titration of the third component was only 70% complete. Hence the terminal portion of the complex curve could be treated as an isolated first order reaction and plotted via the modified Guggenheim method (Kézdy et al. 1958) to yield $\Pi_3$ and $k_3$.

The second component was analyzed in a similar fashion, after subtracting the contribution of the third component.

Finally, the fastest component could be calculated by subtracting the contribution of $\Pi_2$ and $\Pi_3$ from the experimental curve and then plotting this derived curve as a first order reaction.

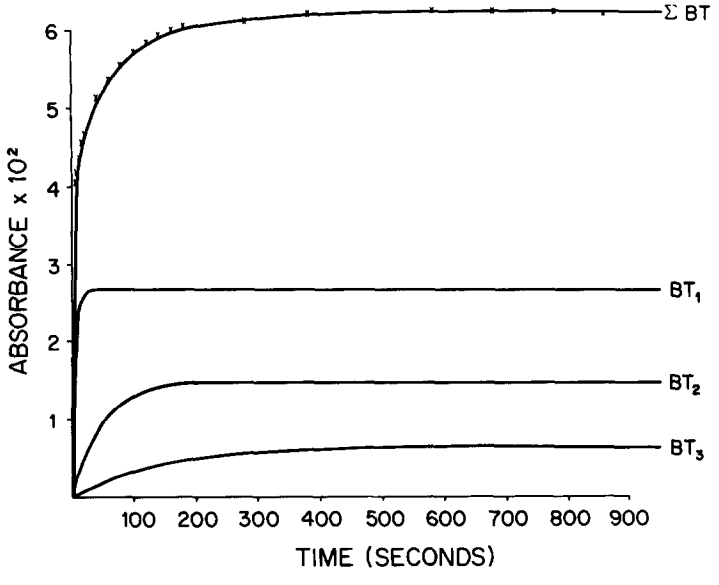
Acrylamide Gel Electrophoresis - The method of Williams and Reisfeld (1964) was used for gel electrophoresis at pH 4.0.

## RESULTS AND DISCUSSION

Titration data with NPGB can yield several important details about enzyme preparations. First, the measurement of the total absorbance change gives the total amount of reactive enzyme present, as indicated by Chase and Shaw (1967). Secondly, information can be obtained about the homogeneity of the enzyme. For example, the titration of Antheraea polyphemus cocoonase obeys pure first order kinetics and this confirms the apparent homogeneity of this enzyme as observed by various physical methods (Kafatos

et al. 1967a). When the measured value of $\pi$ is correlated with the amount and molecular weight (23,000) of protein titrated, it is readily seen that each molecule of cocoonase reacts with one molecule of NPGB.

By contrast, other analogous proteinases do not give simple first order titrations. Nevertheless, their complex kinetics can be analyzed as the sum of several independent first order reactions. For example, once-crystallized porcine trypsin contained two kinetically distinct species in the approximate proportions of 88:12 (Table 1). Furthermore, commercial bovine trypsin consisted of a mixture of at least three distinguishable components in the approximate ratio of 45:35:20. Figure 1 shows the time dependency of p-nitrophenol release from the reaction of unfractionated bovine trypsin with NPGB. Grossly, the curve resembles a first order reaction but no first order reaction could be drawn to fit it as well as the sum of the three theoretical first order curves. Titrations done at pH values ranging from 4 to 8 produced similar results to the one shown in Figure 1. Hence the phenomenon is not an artifact of the pH. Elmore and Smyth (1968a)




Legend to Figure 1

$BT_1$, $BT_2$ and $BT_3$ are the theoretical first order reactions for the first, second and third bovine trypsin components respectively. $\Sigma$ BT is the sum of the theoretical curves plus the zero baseline (.0137 Absorption Units). Experimental points are plotted individually with "x". Rate constants for each bovine trypsin component are: $k_1 = .22$, $k_2 = 0.0202$ and $k_3 = 0.0058$ $sec^{-1}$.

TABLE 1

| FRACTION | BOVINE TRYPSIN | | | α | β | PORCINE TRYPSIN | | A. polyphemus cocoonase | Bovine chymotrypsin |
|---|---|---|---|---|---|---|---|---|---|
| | $BT_1$ | $BT_2$ | $BT_3$ | | | $PT_1$ | $PT_2$ | | |
| $k \times 10^2$ | 20.8* | 2.2±.12 | 0.4±.2 | 2.1±.2 | 21* | 22.6 | 0.2 | 5.7 | .19 |
| % | 45 | 36 | 19 | 100 | 100 | 88 | 12 | 100 | 100 |
| %activity | 74 | | | 55 | 59 | 70 | | 68 | 92 |

Titrations were performed with 50X excess of NPGB titrant in 0.1 M NaAc buffer pH 4.0 and measured at 340 mμ. Standard deviations were calculated from a minimum of three determinations.

* These rates were measured on the Durrum-Gibson stopped-flow spectrophotometer.

titrated bovine trypsin with a different titrant and observed a similar complex acylation kinetics which is consistent with our interpretation. This independent observation shows that the three reactions result from intrinsic trypsin reactivities and are not artifacts of the titrant. In fact, two of the three components of bovine trypsin can be separated by chromatography. The α and β trypsin fractions of Schroeder and Shaw (1968) obeyed simple first order kinetics and are identical to the middle and fast components respectively, of unfractionated bovine trypsin (Table 1). We were unable to locate the slow kinetic component of bovine trypsin among the SE Sephadex fractions. However, all three species have trypsin-like specificity because they are all inhibited by TLCK but not by TPCK.

Acrylamide gel electrophoresis proved useful in examining the results of titration of enzymes with NPGB, because the guanidinobenzoyl enzyme derivatives had a much greater electrophoretic mobility than the parent enzymes. In the case of the SE Sephadex purified enzyme fractions, all of the protein molecules were converted to derivatives. This demonstrates that the purified proteins were fully active and that the acyl enzyme was indeed the product of titration.

In conclusion, we would suggest that titrations with NPGB be done at high and low pH in order to follow the kinetics as well as the total absorption change. Although the extinction coefficient for p-nitrophenol at low pH values is only one third that at high pH, we feel that the information gained about the possible heterogeneity of the protein is well worth the effort. An additional benefit of low pH titrations is the lack of observable spontaneous hydrolysis of NPGB which may occur at higher pH values.

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After submission of this manuscript a paper appeared, Chase, T. and Shaw, E. Biochemistry, 8, 2212(1969), which approached the same problem in a somewhat different manner.

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