Specific Activity of Trypsin

By ROBERT C. PETERSON

Trypsin has been fractionated electrophoretically on cellulose acetate into four zones. The lead zone, which contains the tryptic activity, was treated with specific substrate for enzyme activity and with the Lowry test for protein content. Specific activity of pure trypsin appears to be slightly greater than 8000 N.F. u./mg. Unexpected interference from commercial cellulose acetate was found with the protein test; this was reduced to a practical level. The phenomenon of tailing of protein in electrophoresis was studied.

THE ELECTROPHORETIC heterogeneity of commonly available trypsin is well established (1–13). Investigations in this laboratory on the purity of trypsin suggested the desirability of a simplified method for determining specific activity which could be applied to micro amounts of this enzyme and with slight modification to other enzymes. Electrophoresis on cellulose acetate combined with colorimetry offers such a method.

Earlier work included attempts at quantitative elution from cellulose acetate. It was found that the tryptic activity remaining on the cellulose acetate could be followed with the assay of Schwert and Takenaka (14) using the specific substrate benzoyl arginine ethyl ester (BAEE). From this it was decided that a method for specific activity might be derived from a combined direct determination of protein and tryptic activity in the electrophoretically fractionated zones while on the cellulose acetate. This article reports (*a*) the fractionation of trypsin by cellulose acetate electrophoresis, (*b*) determination of the amount of protein and tryptic activity in the isolated enzyme zone, and (*c*) a calculation of specific activity for the enzyme isolated under these conditions.

Previous similar work includes the extraction or elution of chromatographically or electrophoretically produced zones followed by reaction and measurement (9, 13, 15, 16), the production of color on the strip followed by extraction and measurement (15, 17–21), and direct reaction of the substance in the separated zone without a preceding or following elution (22–27).

Considering the difficulty of total elution, the time needed, and the viability of enzymes, direct test-tube reactions of the excised zones seemed more practical. While fluorigenic substrates (25) or the ninhydrin reaction for amino acids (22–24, 26, 27) have been used in this fashion, the author is not aware of a combination of similar tests being used to determine the specific activity of an enzyme.

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METHODS AND MATERIALS

The standard for all determinations was N.F. trypsin, lot 6040, 3226 N.F. u./mg. (28).

Electrophoresis was carried out in a greatly modified, horizontal cell.[1] Rigidity of components was provided, and evaporation was controlled by supplying inner and outer seals, a slotted inner plate, and by filling air spaces with Lucite blocks and sponges.

Cellulose acetate[2] was selected as the supporting medium. Several brands were tried, and in no case was sufficient precision of mobility found among several strips on a single run for quantitative purposes. Excellent precision was acquired on a single strip, however, by spotting 2 zones at the origin, 0.5 in. from either edge on a 5-cm. wide strip and using one half (a longitudinal cut) as a guide. (See Fig. 1.) The guide strip was dyed for 1 min. when tryptic activity was to be determined; a longer time was permissible when protein was determined. The dye solution was 0.1% Ponceau S in 5% trichloroacetic acid.

Some investigators found that the electrophoretic heterogeneity of trypsin depended to some extent upon the presence of calcium or other ions (2, 4–7, 9) while others did not (11, 13). The greater stability of trypsin in the presence of calcium ions and at an acid pH is well known (29–32). With this in mind, it was decided to use calcium ions in the assay but not during electrophoresis. The buffer was formic acid–pyridine–water/15 of 90%: 2.5: 982.5 by vol., pH 2.65. The strips were prerun 15 min. with reversed polarity before application of sample. The runs were for 3 hr. at room temperature and about 7.8 v./cm. For determination of tryptic activity, the wet strips were used within 1 min. after removal from the chamber; for protein determination, the strips were air dried.

Protein was determined with the Lowry (33) modification of the Folin-Ciocalteu (34) reagent using phenol reagent prepared in this laboratory according to Folin-Ciocalteu (34). The phenol reagent was tested for optimal dilution according to Oyama and Eagle (35), and their Lowry reagent modifications were used. In practice, the protein zone on the cellulose acetate was cut out and placed in a test tube. To this was added 0.5 ml. of water and 2.5 ml. of Lowry's reagent C, and the preparation was allowed to stand exactly 10 min. with frequent agita-

[1] Gordon-Misco, Microchemical Specialties Co.
[2] Oxoid, Colab Laboratories, Inc.

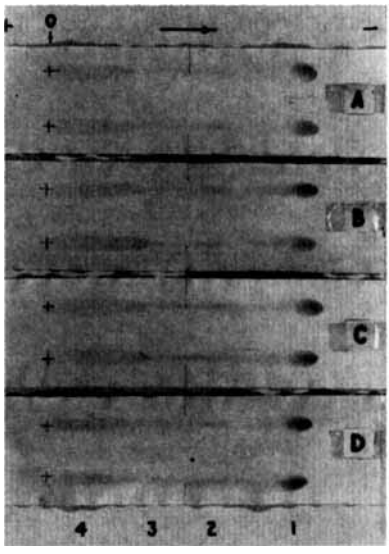

Fig. 1.—Photograph showing resolution and precision of patterns with duplicate spotting on same strip of cellulose acetate. Trypsin, 40 mcg., in aqueous formic acid–pyridine, pH 2.65; application 2.5 in. from anode end. Stained with naphthol blue black, 1% in 7.5% acetic acid. The leading zone contains the tryptic activity. Key: A, Eli Lilly; B, Mann Research Laboratories; C, Nutritional Biochemical Corp.; D, National Formulary.

tion. Folin reagent at the proper dilution, 0.25 ml., was jetted in by syringe and immediately mixed. The tubes were agitated frequently, and the absorption was determined at 1 hr. at 750 mμ with a spectrophotometer.[3] The reference solution was prepared similarly from a blank piece of cellulose acetate cut from the same strip immediately next to the test zone. Early inability to gain precision of results with the Lowry test when trypsin was spotted or electrophoresed on cellulose acetate was found to be due, in great part, to the presence of material in the cellulose acetate, as received, which gave a Lowry-positive test. From experience, it was found that an overnight soak in 0.01 *N* HCl followed by 2 rinses in fresh 0.01 *N* HCl and 2 rinses in deionized water would reduce this Lowry-positive material to usable values.

The Lowry test as described in this report was applied to 5 different lots (strips received at different times were considered different lots) of the cellulose acetate. One strip from each lot was used. Each strip was divided in half; equal sections from each half, with and without the special wash, were submitted to the Lowry test. The results are in Table I. All strips used for the Lowry test in this report were washed as above before being placed in the proper buffer. The contaminant has not been identified.

With each series of protein determinations, freshly dissolved trypsin in water and L-tyrosine[4] [stored in 0.2 *N* HCl, 0.5% by vol. in 37% formaldehyde (36)] were run as standards to assure stability of the Folin reagent.

Benzoyl-DL-arginine-*p*-nitroanilide (BAPA)[4] was

[3] Beckman model DU.
[4] Mann Research Laboratories, Inc.

Table I.—Lowry Protein Test of Cellulose Acetate without Application of Protein and with and without Special Wash to Minimize This Contaminant

	Absorption at 750 mμ			
	Unwashed Strips Sections		Washed Strips Sections	
Lot	1	2	1	2
A	0.095	0.091	0.032	0.025
B	0.196	0.162	0.030	0.030
D	0.089	0.087	0.020	0.017
E	0.079	0.103	0.029	0.034
H	0.087	0.090	0.021	0.022

used as the chromogenic substrate for tryptic activity (37–39). The substrate solution consisted of 100 mg. of BAPA dissolved in 20 ml. of redistilled dimethylsulfoxide and diluted to 100 ml. with pH 8, 0.1 *M* Tris buffer, 0.005 *M* in $CaCl_2 \cdot 2H_2O$. Five milliliters was used for each test; the reaction was carried out for 15 min. at 37° and stopped with 1 ml. of 30% acetic acid. Absorption was measured at 410 mμ *versus* a reference solution containing acetic acid as inhibitor.

RESULTS AND DISCUSSION

Determination of Protein.—The Lowry test was applied to the determination of protein (trypsin) which had been spotted on cellulose acetate under conditions appropriate for, but without, electrophoresis. The linear relationship between absorption values and amounts of protein is shown in Fig. 2. The span of values indicated at each point on the curve is ±1 standard error. Trypsin (30 mcg.) and L-tyrosine (15 mcg.), in solution, were brought through the Lowry test as standards simultaneously with the above series. The daily integrity of the Folin reagent and the test is indicated by the 7 absorbance averages and their standard deviations (trypsin, $\bar{x} = 0.361$, % coefficient of variation = ±1.4; L-tyrosine, $\bar{x} = 0.467$, % coefficient of variation = ±0.8). The plot in Fig. 2 provides a standard for determination of protein in

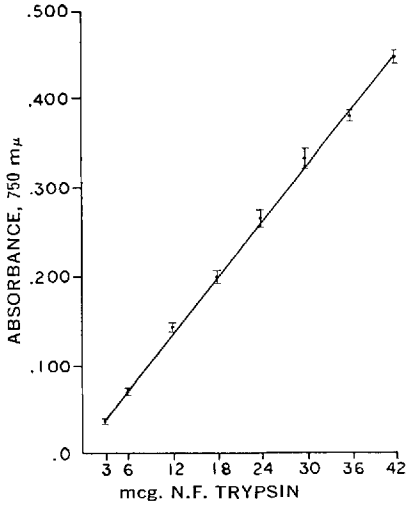

Fig. 2.—Lowry test for protein (trypsin) spotted on cellulose acetate.

the trypsin zone resolved by electrophoresis. Since the Lowry test varies with different proteins, such a curve would have to be constructed for each protein for which such a quantitative procedure would be established.

By incubating the various electrophoresed zones with gelatin, the main part of the tryptic activity was found in the leading zone. Very small amounts of tryptic activity were found all along the strip from the leading zone to the origin; this tailing will be clarified later in this report.

The Lowry test was applied to the lead zone after electrophoresis of varying amounts of N.F. trypsin. For determination of specific activity, only one level is needed, but it was of interest to determine its applicability over the range generally used in cellulose acetate electrophoresis. With the electrophoresis procedure as described, three 5 × 25.4-cm. strips were electrophoresed at one time and used for a single assay. The same amount of enzyme was spotted in duplicate on each strip. A longitudinally cut half served as guide—either after brief dyeing or after partial drying during which the protein zones became opaque and visible. By the latter method a fourth zone can be seen which Ponceau S is too insensitive to stain. Three electrophoretic runs (3 assays) were averaged to give the value at one level of application. Table II gives these values. At the 3-mcg. level there is insufficient protein in the lead zone for application of the test, and, in addition, it can be seen that not until about 18 mcg. is applied is a constant percentage of the applied protein found in the lead zone. This results from the tailing of trypsin activity with a certain saturation of the attracting sites on the cellulose acetate. A plot of applied trypsin against Lowry test absorption from the lead zone is given in Fig. 3. Linearity is found in the range of 18–42 mcg. applied trypsin.

TABLE II.—LOWRY PROTEIN DETERMINATION OF LEAD ZONE AFTER ELECTROPHORESIS OF N.F. TRYPSIN

Applied Trypsin, mcg.	Av. A, 750 mμ	±% Coeff. Var.	Protein[a] in Lead Zone, mcg.	% of Applied Protein in Lead Zone
3	0.002	85		
6	0.019	9	1	16.7
12	0.063	4	5	42
18	0.110	3.5	9.5	53
24	0.144	4.6	12.8	53
30	0.181	4.1	16.3	54
36	0.207	3.3	19	53
42	0.249	3.3	23	55

[a] From standard curve of N.F. trypsin spotted on cellulose acetate.

As with the Lowry tests on trypsin spotted on cellulose acetate, the tests on each level of electrophoresed trypsin were accompanied by standards of trypsin and L-tyrosine. The average absorption values were 0.360 for trypsin (compared to 0.361 before) and 0.465 for L-tyrosine (compared to 0.467 before). The Folin reagent and method have been stable during the course of this work.

Determination of Tryptic Activity.—The chromogenic (BAPA) assay was applied to the determination of enzyme or tryptic activity in zones when various amounts were spotted on cellulose acetate.[2]

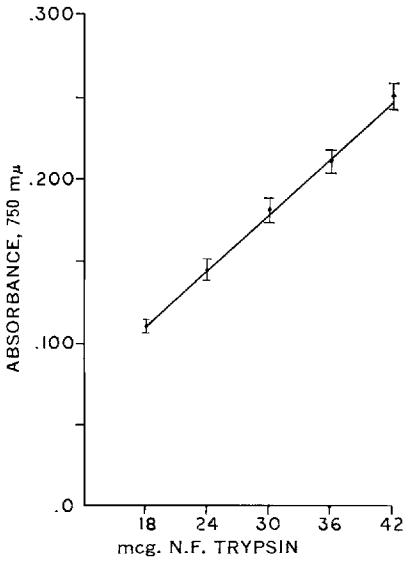

Fig. 3.—Lowry test for protein (trypsin) applied to lead zone (tryptic activity) after electrophoresis. Data are from Table II.

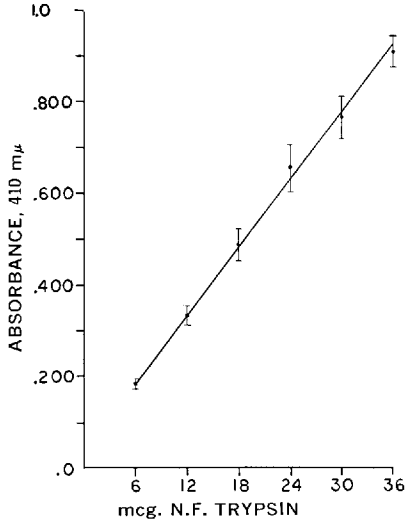

Fig. 4.—BAPA assay of tryptic activity in zones when N.F. trypsin is spotted in various amounts on cellulose acetate.

The cellulose acetate was prepared as for electrophoresis before application. As soon as the applied zone had soaked in, it was cut out and dropped into the reaction solution. Equal-size zones were cut according to a pattern (1 in. wide and 1.5 in. long with length of strip; these corresponded to the enzyme-zone size for the Lowry test), and the time of cutting was staggered every 1 min. A straight line can be drawn to relate the amount of enzyme applied and the average absorption value (Fig. 4).

The BAPA assay was applied to a quantitative study of tailing after electrophoresis of 30 mcg. of N.F. trypsin. The entire pattern of the wet strip was sectioned into 10 equal parts using a dyed guide strip and cardboard template to insure uniformity of zone size. Three electrophoretic runs gave the

average values listed in Table III. The range of absorption values for the lead zone was 0.707–0.720. The average total absorption of all sections was 0.812 with a range of 0.809–0.817. Reasonable agreement of results from application of the BAPA assay to spotted enzyme and to electrophoresed enzyme is indicated by the total absorption values at the 30 mcg. level, 0.763 and 0.812, respectively. N.F. trypsin was used as an indicator of the integrity of BAPA substrate. Ten micrograms in 1 μl. of buffer solution was treated simultaneously with the trypsin zones on cellulose acetate. Indicator values ranged from 0.448 to 0.510 with an average of 0.477; the substrate was satisfactory during the tests. The use of an indicator is necessary since BAPA is not stable indefinitely, even under refrigeration.

TABLE III.—STUDY OF TAILING OF N.F. TRYPSIN DURING ELECTROPHORESIS ON CELLULOSE ACETATE BY MEANS OF BAPA ASSAY

Acetate Section	A, 410 mμ (Tryptic Activity)							
	1,2[a]	3	4	5	6	7	8	9	10
	.712	.031	.018	.012	.009	.009	.008	.007	.006

[a] The absorption values for two equal sections were combined to give the lead zone value.

Since the enzyme activity does not coincide with the dyed protein zones (other than the lead zone), but decreases gradually toward the origin, this is tailing, and zones other than the lead zone do not have trypsin activity. This type of study could be applied to many supporting media in chromatography and electrophoresis in order to determine the influence of the medium on pattern formation.

Determination of Specific Activity.—Following electrophoresis of 30 mcg. of enzyme, the protein in the lead zone was determined with the Lowry test, and the percentage of applied enzyme found in this zone was determined with BAPA. From an assay of N.F. u./mg. of enzyme before electrophoresis the total amount of applied enzyme in N.F. units was determined. The assay values in N.F. u./mg. for these samples before electrophoresis are: *A*, 3226; *B*, 3615; *C*, 3095; *D*, 3565. (See Table IV for coding.) An assumption is made at this point, that since linear plots of concentration *versus* absorbance are obtained with both enzyme assays, a constant ratio exists between the two assays. It is realized that some variation might exist because of a difference in reaction of the N.F. trypsin and the more pure trypsin in the electrophoretic zone, but this is considered to be insignificant. Since in all cases the BAPA assay showed that 87–88% of the applied activity was found in the lead zone, a multiplication of the initial assay in N.F. u./mcg. by the 30 mcg. applied and the per cent recovered, gives the total amount of enzyme found in the lead zone as N.F. units. These totals are found in the second column of Table IV. The specific activity in the lead zone was calculated from these totals and from the totals of protein found in this zone. It appears from these data that the absolute specific activity of pure trypsin is above 8000 N.F. u./mg. The variation in specific activity may be due to the presence of varying amounts of denatured trypsin. All samples were stored as solids under refrigeration, but it has been our experience that occasional degradation is found even under these conditions. It should be pointed out that these samples had been refrigerated for varied amounts of time.

TABLE IV.—SPECIFIC ACTIVITY OF ELECTROPHORETICALLY FRACTIONATED TRYPSIN FROM VARIOUS SOURCES

Source[a]	N.F. u.	Protein, mcg.	Specific Activity, N.F. u./mg.
A	84.2	16.3	5166
B	95.4	11.5	8296
C	81.7	13.8	5920
D	94.1	13.7	6869

[a] A, N.F. lot 6040; B, Eli Lilly, H-1671-B; C, Nutritional Biochem. Corp., lot 2214; D, Mann Research Laboratories lot DA 3566.

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