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# Modifications of Trypsin with Diazobenzenesulphonic Acid

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The modification of the enzymatic activity of trypsin (E.C.3.4.4.4) by diazobenzenesulphonic acid has been studied under various conditions. Amino acid analysis revealed that tyrosine and lysine residues are rapidly modified by covalent coupling to the diazoreagent, whereas histidine residues react slowly. The hydrolytic activity of the enzyme towards *N*-α-benzoyl-DL-arginine-*p*-nitroanilide was influenced only at a high degree of modification of the enzyme structure, while corresponding activity towards *N*-α-benzoyl-L-arginine methyl ester and casein was shown to be much more sensitive to the same chemical modifications. With a thousand-fold excess of diazobenzenesulphonic acid in the coupling procedure the $K_m$ was found to increase 6–7 fold and the $V_{max}$ to decrease 3–4 fold, measuring the interaction between the modified enzyme and *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as substrate. The pH-profile of the modified enzyme was shown to be slightly broader on the alkaline side of the optimum as measured with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as substrate. Butylamine did to some extent protect the active site of the enzyme against the diazoreagent. The heterogeneity of the various modified trypsins could be demonstrated by gel electrophoresis.

The coupling of diazonium-reagents to proteins has a variety of practical applications. Landsteiner [1] showed in the 1930's that it could be a useful tool in immunochemistry. It could also be used to determine the amount of certain amino acids in proteins,[2] to study covalent linkages between proteins and solids or compounds in solution,[3–5] and to study the correlation between structure and function of enzymes.[6–10] Since the $-N=N-$ bond in diazotized proteins could very probably exhibit some semiconductor properties, it is of interest to study semiconductors coupled to enzymes *via* a diazolinkage. Such studies are under way in the authors' laboratory. As a preliminary necessity it was thought that a basic study should be made of how diazoreagents modify enzymes both at the active and at other sites.

The present study of the coupling of trypsin with diazobenzenesulphonic acid is directed towards a wider knowledge of how and where the diazoreagent acts upon an enzyme structure of how one could protect the active site during the coupling procedure, and of how one could determine the number and nature of the side-chains in the protein attacked by the reagent. Enzymatic

constants have been determined for trypsin fully and partially diazotized with DBSA* and compared with those of intact trypsin (as esterase and peptidase). Butylamine, if present during the diazotization procedure, apparently inhibits modifications of the active centre of the enzyme to some degree. The effects of partial modification of the ε-amino groups of lysine are discussed in relation to the pH-profile of the modified enzyme. It was possible to separate diazo-coupled and unmodified trypsin by gel electrophoresis. Modified trypsin, free from native enzyme, has the same activity towards *N*-α-benzoyl-DL-arginine-*p*-nitroanilide as unmodified trypsin.

## MATERIALS AND METHODS

*Materials.* Acrylamide, *N,N'*-methylene bisacrylamide and *N-N-N'-N'*-tetramethylethylenediamine were obtained from Eastman. Casein (Hammarsten grade) was purchased from Merck, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride from British Drug Houses, and *N*-α-benzoyl-L-arginine methyl ester and trypsin (from bovine pancreas: type III, crystallized twice, dialyzed, lyophilized, essentially salt-free) from Sigma Chemicals. Sephadex G-25 fine was obtained from Pharmacia Fine Chemicals. All other chemicals used were of reagent grade or better.

*Diazotization of* p-*aminobenzenesulphonic acid* was carried out according to the method of Saunders.[11] The diazo-compound was collected on a filter, washed with small portions of cold water and 1 g was added to 10 ml cold water in a test-tube and was kept at 0°C. Immediately before use the tube was shaken, the contents filtered, and the clear saturated solution (≈0.05 M) diluted to give the required concentration.

*Coupling of DBSA to trypsin and purification and concentration of the modified enzyme.* Trypsin solution (7.5 ml), in cold 0.04 M trisbuffer pH 8.25, 0.05 M $CaCl_2$, was pipetted into a 25 ml beaker in an ice-bath. The beaker was equipped with stirrer and pH-stat with an automatic titrator (Model TTTIc, Radiometer, Copenhagen). At zero time freshly prepared DBSA-solution (7.5 ml of given concentration) was pipetted into the beaker. The pH-stat maintained the solution at the required pH by adding 0.5 M NaOH (the amount of NaOH used was usually negligible). The reaction mixture was kept at 0°C and constantly stirred.

After a given time an aliquot of 5.0 ml was removed from the reaction mixture, put on a column of Sephadex G25 (50 cm × 2 cm) and eluted (30 ml/h). A Uvicord (type 4701A, LKB) with recorder (set at 280 nm) and collector was used to collect fractions and monitor protein content. The protein fraction was collected and concentrated to 5.0 ml in a diaflo apparatus (UM – 2 filter).

One trypsin blank, taken from the same solution as was used for coupling, was always subjected to the same separation and concentration treatment. Thus it was ensured that the reaction mixture and the blank were comparable.

*Protein concentration* was calculated from UV absorption at 280 nm, assuming for trypsin a specific exctinction coefficient of $E_{280}(1\ \%) = 14.4$ according to Davie and Neurath.[12] All spectrophotometric determinations were made in a Zeiss PMQ II spectrophotometer.

### Enzyme assays

a. *Proteolytic activity.* Proteolytic activity was measured essentially according to the method of Kunitz.[13] The incubation system consisted of 6.0 ml of a 1.5 % casein solution in 0.02 M trisbuffer pH 8.25, 0.025 M $CaCl_2$. The reaction was started by adding 50 μl of enzyme and was incubated at 30 ± 0.1°C for 15 min. The reaction was stopped by adding 3.0 ml of 10 % TCA. After centrifugation the absorbance of the supernatant was measured at 280 nm.

* *Abbreviations used:* BAME = *N*-α-benzoyl-L-arginine methyl ester; BAPA = *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; DSBA = diazobenzenesulphonic acid; TCA = trichloroacetic acid.

b. *Esterase activity.* Activity towards BAME was measured using the method described by Schwert and Takenaka.[14] The reaction mixture consisted of 3.0 ml of 0.3 mM substrate in 0.04 M tris pH 7.50, 0.05 M $CaCl_2$. The reaction was started by adding trypsin (5 $\mu$l) and the absorbance at 253 nm was monitored for 3 min at 30°C.

c. *Peptidase activity.* Activity towards BAPA was determined according to the method of Erlanger *et al.*[15] The reaction was started by adding trypsin (25 $\mu$l) to 3.0 ml of substrate solution (0.2 mM BAPA in 0.04 M tris pH 8.25, 0.05 M $CaCl_2$) in a thermostated cuvette at $30 \pm 0.1$°C, and the absorbance at 410 nm was monitored for 3 min.

*$K_m$ and $V_{max}$ determinations* were carried out with varying concentrations of BAPA, otherwise as described above.

*Inhibition experiments with butylamine.* Butylamine (50 $\mu$l) was added to trypsin (5.0 ml in 0.04 M tris pH 8.25, 0.05 M $CaCl_2$) and pH was adjusted to 9.0 with 6 M HCl. After 10 min of stirring, 5.0 ml DBSA-solution was added and the pH-stat (adjusted to pH 9.0) was started. After 30 min a sample (5.0 ml) was removed and separation and concentration were carried out as described above.

*Amino-acid analyses* were carried out by the analytical division of the Biochemical Institute of Uppsala.

*Polyacrylamide gel electrophoresis.* Gels were prepared in 5 mm glass tubes (inner diameter) according to Hjertén[16] (T 6 %, C 10 %), and were preflushed for 0.5 h in 0.15 M borate buffer pH 6.8, 0.05 M $Ca^{2+}$, and buffer ($5-10$ $\mu$l) containing $15-30$ $\mu$g of enzyme was applied. Both buffer reservoirs were immersed in ice-water. The gels were stained in a solution of coomassie blue in 10 % TCA overnight and was then washed with 10 % TCA.

## RESULTS AND DISCUSSION

*Effect of DBSA concentration, pH, and time in the modification and inactivation of trypsin.* These effects were studied in some preliminary experiments. As can be seen from Table 1, at pH 7.2 there is no decrease in the activity of trypsin towards BAPA after 40 min of modification with a 1000

*Table 1.* The influence of time and pH on the deactivation of trypsin by DBSA, as measured with BAPA as substrate. Trypsin conc.:DBSA conc. 1:1000.

| Modification time (min) | % Activity | | |
|---|---|---|---|
| | pH 7.2 | pH 8.2 | pH 9.2 |
| 20 | 100 | 81 | 8 |
| 40 | 100 | 60 | 5 |

fold excess of DBSA. However, the reactivity of DBSA is greatly increased at higher pH, resulting in an increased modification of trypsin with a corresponding decrease in the activity towards BAPA. Thus after 40 min of modification at pH 9.2 the activity towards BAPA was only 5 % of that of unmodified trypsin. As can also be seen from Table 1 doubling the time (20 min to 40 min) does not seem to markedly influence the deactivation. The influence of time was more carefully investigated by modification of trypsin with 1000 fold excess of DBSA at pH 8.2 over 24 h. As can be seen from Fig. 1 the activity rapidly decreases to reach a level of $4-5$ % after about 10 h.

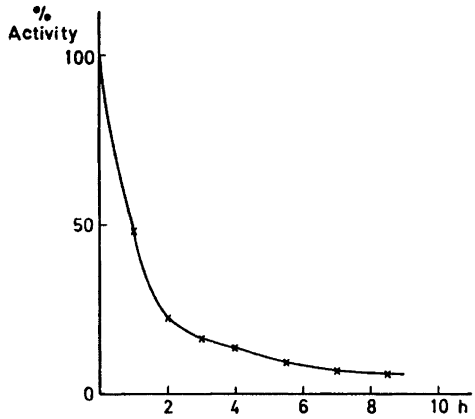


*Fig. 1.* The decrease in activity towards BAPA of trypsin modified with 1000 fold excess of DBSA at pH 8.2. The figure shows the first 10 h, after which the activity was essentially constant (4 – 5 %) during additionally 14 h.

This activity is kept constant throughout the experiment. The influence of varying DBSA-concentrations was investigated at pH 9.2 during 40 min of modification. There proved to be remarkably little activity towards BAME although that towards BAPA was little affected (Table 2) except at high degrees of enzyme modification.

*Table 2.* Deactivation of trypsin by DBSA of varying concentrations during 40 min at pH 9.2.

| Mol. prop. Trypsin:DBSA | 1:1 % | 1:10 % | 1:100 % | 1:1000 % |
|---|---|---|---|---|
| BAPA activity | 100 | 100 | 75 | 3 |
| BAME activity | 100 | 80 | 20 | 0 |

*Effect of $NO_2^-$ and sulphanilic acid on trypsin at pH 8.25.* The possibility could not be excluded that $NO_2^-$ and sulphanilic acid, probably present in very small amounts in the diazo-solution, might influence the activity of trypsin. Thus the activity of a solution containing trypsin was compared with that of (a) trypsin and 1 % sulphanilic acid, and (b) trypsin and 1 % $NaNO_2$ (all with 3 mg trypsin/ml 0.04 M tris pH 8.25, 0.05 M $CaCl_2$) after 24 h incubation at 4°C. No changes in activity towards BAME, BAPA, and casein could be detected. As comparison we should mention that Gundlach *et al.*[10] did not find any deamination of lysine residues in chymotrypsin after treatment with $NO_2^-$ at pH 10.3.

On the basis of these results a new series of experiments was instigated. Trypsin was treated with DBSA at pH 9.0 in the molar proportions 1:1, 1:10, 1:100 and 1:000, and at each concentration one sample was removed after 0.5 h and one after 3 h. The activities towards BAME, BAPA, and casein were

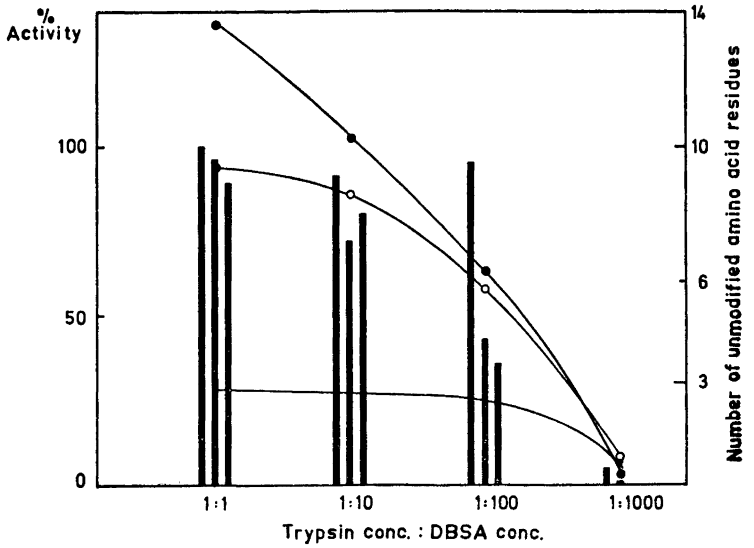


*Fig. 2.* Results of 3 h modifications. From left to right, the activities towards BAPA, BAME, and casein are compared with the number of unmodified histidyl ( × ), lysyl ( ● ), and tyrosyl residues (○). While the modification process can be regarded as continuous the deactivation process cannot surely be regarded so, and is thus represented by pile diagrams. Unmodified trypsin contains 3 histidyl, 14 lysyl and 10 tyrosyl residues, and the activities towards BAME, BAPA, and casein are 100 %.

determined. There proved to be appreciable differences between 0.5 h and 3 h only at the 1:1000 modification. As can be seen in Fig. 2 there are only small differences between the decreases in activity towards BAME and casein. The activity towards BAPA is much higher and only affected at very high concentrations of DBSA, as noted in the preceding experiments.

*Amino acid analyses* revealed that only histidyl, lysyl, and tyrosyl residues had been modified to any appreciable extent, which is well in agreement with the results of Tabachnick.[17] As can be seen from Fig. 2 lysyl and tyrosyl residues are most susceptible to modification. The low reactivity of the histidyl residues is interesting. Gundlach *et al.*[10] have found a marked retardation of the reaction rate of DBSA with the histidyl residues of chymotrypsin.

*Protection of active site with butylamine.* Inagami and Murachi [18] have reported that butylamine is a strong competitive inhibitor of trypsin. We wanted to examine the possible protection of the active site of trypsin by treating it with butylamine and then add DBSA. Though Howard and Wild [19] report that butylamine reacts with diazonium compounds to a slight degree, it seems as if butylamine partly protected a certain region of the enzyme from modification. From Table 3 can be seen, that although the "protected" trypsin has 0.4 histidine, 2.5 lysine, and 0.9 tyrosine residues more modified than the "1:10" modification they have the same activities. This can only be explained by assuming that butylamine has protected the active site to a certain degree and that other residues apart from this have been modified.

*Table 3.* Comparison of some amino acid residues and some activities of modified trypsins, one of them protected with butylamine.

| Trypsin conc.: DBSA conc. | | 1:10 | 1:100 | 1:1000 | 1:1000 |
|---|---|---|---|---|---|
| Relative conc. of butylamine | | 0 | 0 | 0 | 2000 |
| Time for modification (h) | | 3.0 | 0.5 | 0.5 | 0.5 |
| Amino acid residues (unmod.) | His | 2.7 | 2.5 | 1.2 | 2.3 |
| | Lys | 10.3 | 6.6 | 0.7 | 7.8 |
| | Tyr | 8.6 | 5.7 | 2.0 | 6.7 |
| % Relative activity towards BAME | | 72 | 50 | 4.5 | 73 |
| % Relative activity towards BAPA | | 91 | 99 | 9 | 100 |
| % Relative activity towards casein | | 80 | 50 | 0 | 80 |

While the decreases in activity towards BAME and casein are quite continuous, the decrease in activity towards BAPA, from 95 % at the 1:100 to 5 % at the 1:1000 modifications, is abrupt. This could indicate that a modification is taking place near the active site, for example at 59-tyr or 60-lys.[20] This means that it could be more difficult for the substrates to enter the active site. For BAME the binding step is more rate limiting than for BAPA.[21] Thus a somewhat greater difficulty to enter the active site would not affect the activity towards BAPA as much as BAME. The above mentioned abrupt decrease in activity towards BAPA could for example be explained by assuming that 57-his has been modified. As can be seen in Fig. 2, the histidine residues are rapidly modified between 1:100 and 1:1000. Even the results from the active-site-protected trypsin seem to make this probable. This has, however, to be proved by analyses of the region around 57-his.

Casein is a very big molecule, compared to BAME and BAPA. This could mean that steric factors other than those which affect BAME and BAPA can be involved. Otherwise it could be expected that casein should react like BAPA.

$K_m$ and $V_{max}$ for the activity towards BAPA were determined for all the modifications. There was only a significant difference, as would be expected from Fig. 2, at the modification with 1000 parts excess of DBSA, when $K_m$ increases 6 – 7 times while $V_{max}$ decreases 3 – 4 times.

*The pH-profile* for the activity towards BAPA proved to be a little broader at the alkaline side of the optimum for the modified trypsins. This was most apparent at the 1:10 modifications. Though it is just a small difference, it is interesting to know that similar differences, though more striking, have been reported upon modification of the ε-amino groups in trypsin. According to Terminiello *et al.*[22] acetylation of the amino groups in trypsin shifts the pH-optimum of the enzyme, using BAEE as a substrate, towards a higher pH. This is also pointed out by Labouesse and Gervais.[23] Maekawa and Liener [24] reported that on glycosylamidylation (Ga) of trypsin "the pH-optimum of Ga trypsin was broader and somewhat more alkaline than that of trypsin". In the latter case three lysyl and three histidyl residues were thought to have been modified. It thus seems that a neutralization, partial or total, of the ε-amino groups of lysine should cause a change in the pH of the micro-environment of trypsin. Thus it is necessary with a more alkaline pH to get the same dissociations of the functional groups necessary for optimal activity.

*Heterogeneity of the coupling products.* Among others Houston and Walsh [25] have pointed out that "it is difficult to interpret the effects of chemical modification upon reaction velocities without distinguishing between the case that the chemical modification has completely inhibited a proportion of the enzyme molecules and the case that all of the enzyme molecules are partially inhibited". Since modification with DBSA means both introduction of a negative group and partial neutralization of the positive charge of the ε-amino groups, unmodified trypsin can be distinguished on the bases of the difference in the respective isoelectrical points. Thus separation of modified and unmodified enzyme was achieved by gel electrophoresis.

While we were unable to observed any difference in the gel electrophoretic pattern between unmodified trypsin and 1:1 modified, four new bands as well as the originals were seen at 1:10 modification. At the 1:100 and 1:1000 modifications only one band could be detected with no traces of unmodified trypsin.

It can thus be stated that all the fractions of the 1:10 modification are active, which, judging from the high activity towards BAPA of the 1:100 modification, should even here be the case. It is, however, very probable that the extensive modification 1:1000, 3 h, should have some fraction(s) not being active at all.

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