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The Action of Enterokinase on Trypsinogen *

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Using Edman's phenyl*iso*thiocyanate method, valine and *iso*leucine were identified as the N-terminal amino acids of trypsinogen and of trypsin formed by autoactivation, respectively.

The activation of trypsinogen by enterokinase was followed by determining the N-terminal amino acids. *Iso*leucine was detected in increasing quantity during the course of the activation, the amount being directly related to the appearance of tryptic activity.

Enterokinase seems to be a peptidase with a high specificity for trypsinogen.

By means of kinetic studies Kunitz [2] showed that enterokinase is an enzyme which activates trypsinogen to trypsin. No studies have, however, been carried out on the chemical nature of the reaction.

Davie and Neurath [3] investigated the mechanism of autoactivation, the activation by trypsin itself, and showed that a valylpeptide was split off, the amount being proportional to the tryptic activity developed. Thus trypsinogen is activated to trypsin by means of a limited hydrolysis giving rise to a new N-terminal amino acid.

As to the N-terminal amino acids of trypsinogen and of trypsin formed by autoactivation, Rovery, Fabre and Desnuelle [4] have identified valine as the N-terminal amino acid of trypsinogen by using Sanger's dinitrofluorobenzene method, and *iso*leucine as that of DIP-trypsin (di*iso*propylphosphoryl trypsin) by using both Sanger's method and Edman's phenyl*iso*thiocyanate method.

Thus, if enterokinase acts as a peptidase on trypsinogen as trypsin does, the reaction may be followed by determining free amino groups which are liberated during the hydrolysis of peptide bonds. The present paper will show that this is the case.

In the first instance, the N-terminal amino acids of trypsinogen and of trypsin formed by autoactivation were determined by Edman's method and results were obtained which confirm the findings already mentioned. The

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activation by enterokinase was then followed by determining the N-terminal amino acids. *Iso*leucine was detected in increasing amount paralleling the appearance of tryptic activity.

EXPERIMENTAL

Trypsinogen was prepared from beef pancreas according to the method of Northrop and Kunitz [5]. The crystalline product was purified further by treatment with trichloroacetic acid. Enterokinase was prepared from swine duodenal fluid contents according to the author's method [6,7]. It has an activity of 2 000 units per mg dry substance, which indicates a high degree of purity. The estimation of the tryptic activity was performed according to Anson's hemoglobin method [5].

The N-terminal amino acid of trypsinogen. Rovery, Fabre and Desnuelle [4] have detected valine as the N-terminal amino acid of trypsinogen by using the dinitrofluorobenzene (DNFB) method. However, alkaline conditions under which the coupling between protein and DNFB takes place are quite favourable to the autoactivation and autodigestion due to an unavoidable trace of trypsin present in the trypsinogen preparation.

Several amino acids including valine and *iso*leucine were in fact detected when a non-denatured and soluble trypsinogen preparation was used instead of the denatured and insoluble one used by the French workers. The pre-treatment of the trypsinogen preparation by di*iso*propyl fluorophosphate (DFP) eliminated amino acids other than valine.

The use of Edman's method simplified the situation. By this method, both DFP-treated and non-treated trypsinogen preparations gave, due to the rapid reaction between phenyl*iso*thiocyanate and the terminal amino group as demonstrated by Edman [8,9], only one single amino acid, identified as valine.

The N-terminal amino acid of trypsin formed by autoactivation. The trypsinogen preparation was activated at 0° C and pH 5.6 using 0.1 M triethylammonium acetate buffer which contains 0.05 M $CaCl_2$. 10 mg of trypsinogen was dissolved per ml, giving a concentration of trypsin of 0.05 mg per ml without any further addition of pure trypsin. Under these conditions 100 % activation could be obtained after 22 hours. The reaction mixture was then adjusted to pH 2.5 by the addition of 1 N HCl. Aliquots were taken, diluted with water and to these solutions an equal volume of pyridine was added. To 2 ml of the pyridine-water solutions containing trypsin corresponding to 4 mg trypsinogen, 20 μl of phenyl*iso*thiocyanate was added with vigorous stirring. The pH was then adjusted to 9.0 by the addition of a few drops of triethylamine. The reaction was allowed to continue for two hours at room temperature with occasional stirring and the further addition of triethylamine in order to keep the pH between 8.5 and 9.0. After the completion of the coupling, the reaction mixture was washed with benzene (3 × 5 ml) in order to remove pyridine and an excess of phenyl*iso*thiocyanate. To the aqueous layer, 8 ml of acetone was added and the precipitate of phenylthiocarbamyl derivatives was collected by centrifugation and dried *in vacuo*. The dried material was then suspended in 1 ml of 1 N HCl and heated for one hour in boiling water with occasional stirring. The phenylthiohydantoin derivatives of the amino acids were then extracted with ethyl acetate (3 × 2 ml) and the combined extracts were washed with 0.8 ml of water. 2.0 ml of the extract was diluted to 5 ml with 95 % ethanol and the ultraviolet absorption measured. From the shape of the curve, the formation of phenylthiohydantoin derivatives of amino acids was confirmed. The yield, referred to the amount of trypsinogen, was 170 % based on the optical density at 269 mμ. For the molecular weight of trypsinogen and the molecular extinction coefficient of phenylthiohydantoin derivatives of the amino acid, 23 800 and 16 000 were used, respectively.

The rest of the extract was evaporated to dryness, dissolved in ethylene chloride and aliquots used in the identification experiments.

For this purpose paper chromatography was used according to the directions of Sjöquist and Edman [10,11]. A starch-treated Whatman No. 1 paper was dipped into a mixture of acetone and formamide (3 : 1 by volume) and dried at room temperature until the acetone had evaporated. The test solution was then applied to the paper and developed with xylene. Two spots, derived from valine and *iso*leucine, were observed either by

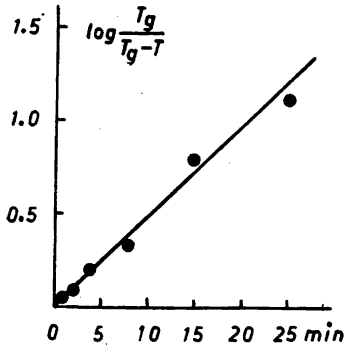

Fig. 1. Reaction of enterokinase at high trypsinogen concentration. T_g: Initial concentration of trypsinogen. T: Tryptic activity at any time. The line shows the theoretical relationship for a first order reaction and the points are the experimental values obtained.

ultraviolet absorption with the aid of a fluorescence screen or by spraying with the KI-NaN_3 reagent. Since no spots other than these two were observed, it is evident that *iso*leucine is an N-terminal amino acid of trypsin formed by autoactivation.

The N-terminal amino acid of trypsin formed by the action of enterokinase. The experimental conditions cause some difficulties in the study of the activation of trypsinogen by means of enterokinase. The standard conditions for the estimation of enterokinase activity established by Kunitz are quite inconvenient for the present purpose because of the extremely low concentration of trypsinogen (65 μg per ml). The use of a high concentration of trypsinogen is accompanied by autoactivation, autodigestion or formation of inert protein due to trypsin. In this case, the kinetics do not correspond to those of a first order reaction as has been thoroughly demonstrated by Kunitz [5].

These disadvantages could be overcome by the use of a high concentration of enterokinase together with the use of triethylammonium acetate buffer which seems to stabilize trypsin. In a series of test tubes, 5 mg of trypsinogen and 0.8 mg of enterokinase preparation having an activity of 2 000 units per mg were dissolved in 12.5 ml of 0.1 M triethylammonium acetate buffer (pH 5.6). The reaction was carried out at 0° C. Under these conditions it corresponds to a first order reaction as shown in Fig. 1, which indicates that there are no appreciable signs of autoactivation or autodigestion.

The activation was stopped at intervals by adding 1 ml of 1 N HCl to 12.5 ml of reaction mixture. To this acid solution an equal volume of pyridine, containing phenyl-*iso*thiocyanate in a concentration of 50 μl per ml was added. The pH was then adjusted to 9.0 by the addition of a few drops of triethylamine.

As to the stability of trypsin in pyridine-water (1:1) in which the coupling reaction takes place, it was found that 60 % of the activity is recovered after 2 hours at room temperature. When both trypsinogen and trypsin were present in an equal concentration of 0.1 mg per ml in this solvent, corresponding to 50 % activation where the autoactivation shows the highest velocity, 20 % of trypsinogen was converted to trypsin after 2 hours at room temperature. However, when phenyl*iso*thiocyanate was present in the above-mentioned concentration, tryptic activity was very quickly blocked, 95 % of the activity being lost 5 min. after the addition of pyridine containing phenyl*iso*thiocyanate. Consequently, the risk of autodigestion or autoactivation due to trypsin under the coupling conditions could be excluded.

The coupling was continued for 2 hours at room temperature, the pH being maintained between 8.5 and 9.0 by occasional addition of triethylamine. The reaction mixture was then washed with benzene (5 × 20 ml) and the aqueous layer was then lyophilized. The dried material was suspended in 3 ml of 1 N HCl and heated at 100° C for one hour. The phenylthiohydantoin derivatives of the amino acids were extracted with ethyl acetate (3 × 2 ml). The combined extracts were then washed by 0.8 ml of water and evaporated to dryness. The residue was dissolved in 40 μl of ethylene chloride and 5 μl of the solution was used for a single paper chromatography run.

The paper chromatogram which was prepared as described above and made visible by spraying with KI-NaN_3 reagent is shown in Fig. 2. In this figure, the amount of valine

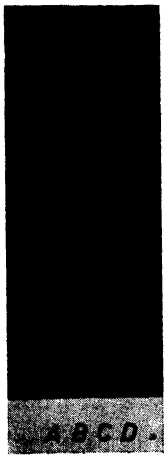

Fig. 2. Paper chromatogram demonstrating the development of isoleucine *as a new N-terminal amino acid during the activation. A: Standard substances, phenylthiohydantoins of valine and* isoleucine. *B: Zero time activation. C: 38 % activation after 4 minutes. D: 79 % activation after 15 minutes.*

remains almost constant throughout the whole course of activation whereas the amount of *iso*leucine increases with the progress of activation. The spots localized at the original point and those of low R_F values were considered to have no relation to the activation reaction.

For the quantitative evaluation of the developed spots on the chromatogram, the elution technique was applied. Thus, the spots identified by ultraviolet absorption with the aid of a fluorescence screen were cut off, eluted with 1 ml of 95 % ethanol for one hour at room temperature and the ultraviolet absorption at 269 mμ determined. The amount of valine was practically constant and that of *iso*leucine increased throughout the activation process. Since the phenylthiohydantoin derivatives of valine, *iso*leucine and alanine can be eluted just as effectively (about 80 % of the substance applied on the paper [12]), the amounts of *iso*leucine were corrected with reference to those of valine in each run. In Fig. 3, the percentages of *iso*leucine found and corrected are shown at each step of activation.

It could be concluded that there is a quantitative relationship between the degree of the activation of trypsinogen caused by enterokinase and the amount of *iso*leucine appearing as N-terminal amino acid.

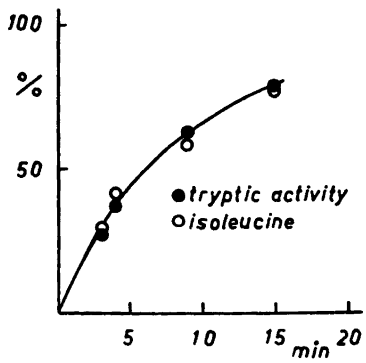

Fig. 3. Relation between the percentages of isoleucine *as referred to valine and those of activation.*

DISCUSSION

Since valine and *iso*leucine are the N-terminal amino acids of trypsinogen and trypsin, respectively, as found by Rovery *et al.*[4] and by the present author, the appearance of *iso*leucine as N-terminal amino acid and the constant presence of valine suggest that enterokinase acts as a peptidase splitting off a valylpeptide from the amino end of trypsinogen, a reaction similar to that in the case of autoactivation. The good agreement of the percentages of *iso*-leucine referred to valine and those of activation throughout the whole activation process excludes the possibility of a transitory formation of an active form of trypsinogen.

Although enterokinase acts as a peptidase in this respect its action is highly specific. Since trypsin acts on esters of lysine and arginine, the activity of the enterokinase preparation was tested on tosylarginine methyl ester and lysin ethyl ester. A slight activity was observed at a pH of between 6 and 9. Judging from the low activity and the peak of the activity curve at pH 7.5 it is very likely that the activity was caused by a contamination by trypsin.

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