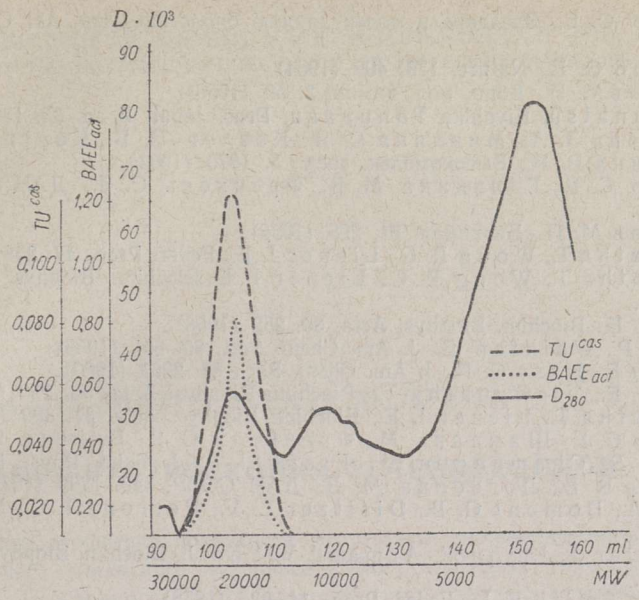
EESTI NSV TEADUSTE AKADEEMIA TOIMETISED. XV KÖIDE
FÜÜSIKA-MATEMAATIKA- JA TEHNIKATEADUSTE SEERIA. 1966, NR. 4

ИЗВЕСТИЯ АКАДЕМИИ НАУК ЭСТОНСКОЙ ССР. ТОМ XV
СЕРИЯ ФИЗИКО-МАТЕМАТИЧЕСКИХ И ТЕХНИЧЕСКИХ НАУК. 1966, № 4

O. KIRRET, E. LIPPMAA, I. ARRO, H. HEINLO

THE ACTIVE SITE AND ACTIVE FRAGMENTS OF ENZYMES. I

It has generally been assumed that the most important role of regions outside the active site of any non-allosteric enzyme is that of a carcass and that some enzymes can be degraded to active fragments with a lower molecular weight [1–6]. Such fragments have been prepared from various enzymes, notably from pepsin [7–10], trypsin [11–24, 44], papain [25–31], ribonuclease [32–38], aldolase [39], enolase [40, 41], and leucine aminopeptidase [42]. These results are compatible with all ideas about the mechanism of enzyme action [6, 43, 46] insofar as the fragments are big enough to have tertiary structure. But several laboratories have reported the preparation of active fragments with molecular weights below 10000 [9, 13, 14, 18, 19, 28, 29, 31] and even below 3000 [6, 11, 22]. The last-mentioned fragments are practically pure active sites and serious conclusions have been drawn from their existence. Yet even in 1960 Desnouelle and later Chevallier were not able to prepare active fragments from trypsin [3, p. 131; 24]. The selective degradation experiments with papain [25–29] have proven wrong [30]. Clearly further study of enzyme fragments is warranted because of the fundamental importance of this problem and since much better enzyme preparations and separation methods are now available. In many cases dialysis has been used to separate the fragments. Although some separation can be achieved [45], small amounts of quite high-molecular fractions and even native enzymes can penetrate the membranes. Gel filtration [47] is a much better method for protein separation according to molecular weights [47–51]. If the gel column is well equilibrated, one can be reasonably sure that no high-molecular fractions can be found in the fragment fractions. Since the smallest and thus the most important active fragments were prepared by the autolysis of trypsin, this enzyme was studied first. Our experiments were carried out with commercial crystalline trypsin (Spofa, lot № 050655). Since the origin of the fragments was not very important in this investigation, the enzyme was used as received without preliminary purification. But the high molecular weight part of the sample appeared to be chromatographically homogeneous. To prevent contamination with other enzymes, trypsin was degraded by autolysis at 35° C in 0.1 m formiate buffer (pH 3.2, ionic strength 0.5) for 160 h and in unbuffered water solution at pH 7.8 to 7 for 3.5 h. In both cases residual activity was about 10% from the initial value. The reaction products were separated by gel filtration on a thermostatted Sephadex G-100 column (1.1×140 cm) at 20° C in 0.1 m formiate buffer (pH 3.2, ionic strength 0.5). 1 ml fractions were taken with an automatic fraction collector and protein content was measured at 280 mμ. Hammarsten casein (CAS) and N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) were used for the assay of tryptic activity [52]. The results of slow



degradation at pH 3.2 (50 mg of trypsin in 2 ml of buffer) are presented graphically. The enzymic activity of all fractions was measured, but only the trypsin peak at 104 ml showed proteolytic (CAS) and esterase (BAEE) activity, in all other cases the activity figures were below the error limit (±5%). Practically no activity was lost in the separation process, total activity of the collected fractions being equal to the activity of autolysis products before separation. The error never exceeded ±10% and was considerably less with BAEE as the substrate. Although the trypsin peak may contain some modified enzyme, no active fragments with molecular weights below 15000 were formed. The extrapolated molecular weights of the main fragment fractions, using ribonuclease for calibration in this determination [51], were 12000 and 4000. The lower molecular weights are not very accurate.

Quick autolysis of trypsin was run in water at pH 7.8 to 7. 50 mg of trypsin was dissolved in 2 ml of distilled water containing traces of NaOH (initial pH 7.8). The solution was incubated at 35° C for 3.5 h, mixed with 2 ml of 0.2 m formiate buffer (pH 3.2, ionic strength 1.0) and centrifuged. The autolysis products were separated by gel filtration as described above. The results were essentially identical with the results of slow autolysis. No active fragments were found.

It appears that nearly the whole trypsin molecule, or in any case more than $^5/_8$ of it takes an active part in enzymic catalysis. One cannot prepare "pure active centres" of trypsin by autolysis.

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Academy of Sciences of the Estonian SSR,
Institute of Chemistry and Institute of Cybernetics

Received
Sept. 24, 1966

O. KIRRET, E. LIPPMAA, I. ARRO, H. HEINLO

FERMENTIDE AKTIIVNE TSENTER JA AKTIIVSED FRAGMENDID. I

О. КИРРЕТ, Э. ЛИППМАА, И. АРРО, Х. ХЕЙНЛО

АКТИВНЫЙ ЦЕНТР И АКТИВНЫЕ ФРАГМЕНТЫ ФЕРМЕНТОВ. I