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Water-insoluble enzymes for peptide sequencing: Dipeptidyl aminopeptidase I (cathepsin C), an enzyme with subunit structure

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SUMMARY

Dipeptidyl aminopeptidase I (cathepsin C, EC 3.4.4.9) from bovine spleen or rat liver, which consists of eight subunits and cleaves dipeptides sequentially from the N-terminus of polypeptides, has been coupled to insoluble supports with retention of transferase, amidase and peptidase activity, demonstrated by assay and by degradation of the peptide hormone analogue, tetracosactrin. Attachment of the enzyme to the polymer Enzacryl Polythiolactone yielded preparations of highest activity. This may be associated with the presence of thiol groups in the polymer matrix, liberated during the coupling reaction, since dipeptidyl aminopeptidase I has a thiol requirement. There is no apparent change in pH optimum as a result of coupling.

The unique peptidase properties of cathepsin C (EC 3.4.4.9) have become apparent recently as a result of extensive studies by McDonald and co-workers who have shown[1,2] that the enzyme, renamed dipeptidyl aminopeptidase I, cleaves dipeptides sequentially from the N-terminus of polypeptide chains. The enzyme has requirements for chloride and thiol. It has broad specificity and only peptides with N-terminal lysine or arginine or with proline as the second or third residue from the N-terminus are considered resistant to degradation. The potential value of dipeptidyl aminopeptidase I in peptide sequencing arises from the large number of bonds broken [the number in the oxidized insulin B chain, 13, equals the total cleaved by trypsin (EC 3.4.4.4), chymotrypsin (EC 3.4.4.5) and pepsin (EC 3.4.4.1)], and from the ability to obtain a second overlapping set of dipeptides by cleavage of a chain from which the N-terminal residue has been removed selectively[3–5], which will usually permit unambiguous assignment of sequence if the dipeptides can be identified. The availability of water-insoluble enzymes, which are

more stable than native enzymes (*e.g.* ref. 6), has stimulated several groups to investigate the properties of multi-enzyme systems[7–9]. In particular, the use by Bennett *et al.*[9] of Sepharose-bound peptidases and our (unpublished) results with insoluble trypsin, thermolysin and chymotrypsin have shown that polypeptides are readily degraded by such systems with minimal contamination. We have investigated the covalent attachment of dipeptidyl aminopeptidase I, which consists of eight subunits arranged in two tetrameric groups[10], to a number of water-insoluble polymers and the peptidase activity of these preparations as a preliminary to their incorporation in a multi-enzyme system for sequence studies. This paper describes some of our results.

Dipeptidyl aminopeptidase I was isolated from bovine spleen or rat liver by the method of Metrione *et al.*[10] as adapted by McDonald *et al.*[1] for isolation of the rat liver enzyme. Dipeptidyl aminopeptidase I was assayed by the transferase method of Metrione *et al.*[11], three transferase units are approximately equal to one arylamidase unit determined by fluorimetric assay[1]. We confirmed that the yield and purity of dipeptidyl aminopeptidase I obtained from rat liver were greater than from bovine spleen[1] and most of our experiments have employed the liver enzyme. Purification to the Sephadex G-200 stage gave enzyme of sufficient purity and adequate specific activity for use in sequence studies, although a minor contaminant was observed on polyacrylamide gel electrophoresis at pH 8.3. McDonald *et al.*[12] have recently shown that treatment of the Sephadex G-200 fraction with DFP and EDTA destroys the activity of some contaminating enzymes.

Details of the water-insoluble polymers are given in Table I together with methods of activation and reactive groups on the enzyme. The coupling reactions were carried out in accordance with the suppliers' instructions. The following procedure was employed with Enzacryl Polythiolactone. To the polymer (40 mg) in 0.1 M phosphate buffer (pH 6.2; 5 ml) was added dipeptidyl aminopeptidase I (1.5 ml, 94 units in 3.2 mg protein, previously dialysed against the same buffer) and the mixture stirred for 5 h at 4°C. The enzyme preparation was washed carefully three times each with 0.2 M acetate buffer (pH 5.0) and the same buffer containing 1 M sucrose and 1 M NaCl to remove unbound enzyme, then with increasing concentrations of acetone in water, dried and stored over silica gel at 4°C. Insolubilized dipeptidyl aminopeptidase I was assayed as above but the

TABLE I

WATER-INSOLUBLE POLYMERS TO WHICH DIPEPTIDYL AMINOPEPTIDASE I HAS BEEN COUPLED, TOGETHER WITH THE TRANSFERASE ACTIVITIES OF THE PRODUCTS[11]

*Supplier** *and polymer*	*Method of activation*	*Group in dipeptidyl aminopeptidase I which couples*	*Transferase activity of product (units/min per mg polymer)*
P Sepharose 4 B	CNBr	$-NH_2$	0.001
K Enzacryl Polythiolactone	None	$-OH + -NH_2$	0.015
K Enzacryl AA	Diazotization	–OH of Tyr + aromatic rings	0.0061
	$CsCl_2$	$-NH_2$	0.0057
K Enzacryl AH	Diazotization	$-NH_2$	Not detected

*P: Pharmacia, London, W.5.; K: Koch-Light, Colnbrook.

mixture was stirred for 1 h instead of 10 min. Analysis of the washings showed that 67% of available protein was bound to Polythiolactone polymer and this retained 3.0% of the expected activity. Table I shows the activity of each type of preparation of insoluble dipeptidyl aminopeptidase I expressed per mg polymer to show the relative overall efficiency of coupling. It is clear that the Enzacryl Polythiolactone preparation, which in part involves coupling through hydroxyl, retains higher activity than the others. The insoluble enzyme was stable on storage at 4°C whereas soluble dipeptidyl aminopeptidase I lost approx. 10% activity per month under these conditions.

The pH optimum was studied by the arylamidase assay of Gorter and Gruber[13] modified as follows: reaction mixtures containing 0.77 mg Gly–Phe-*p*-nitroanilide were incubated at 37°C, the buffers were 0.1 M citric acid–0.2 M Na_2HPO_4 in the range pH 3–8, and the ionic concentration was not adjusted. The absorbance was read after 10 min in experiments with soluble dipeptidyl aminopeptidase I and after 2 h, after centrifugation, in those with insolubilized dipeptidyl aminopeptidase I. Fig. 1 shows that no significant shift in pH optimum has taken place as a result of insolubilization.

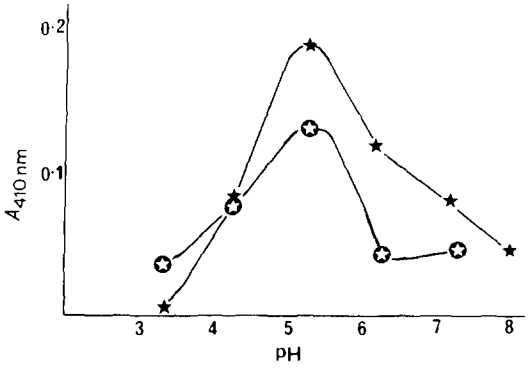

Fig. 1. pH profile of amidase activity of soluble dipeptidyl aminopeptidase I and insoluble Enzacryl Polythiolactone–dipeptidyl aminopeptidase I using Gly–Phe-*p*-nitroanilide as substrate[13], as modified in text. Solid stars, soluble enzyme; stars in solid circles, insoluble enzyme.

Tetracosactrin (β1–24 ACTH, Synacthen), a generous gift from Dr G.P. Lewis, CIBA Laboratories, Horsham, was degraded under the following conditions:
(a) Experiments with soluble dipeptidyl aminopeptidase I (about 1.4 units) were carried out at pH 6.0[1] with 1 mg tetracosactrin. (b) A suspension of Enzacryl Polythiolactone–dipeptidyl aminopeptidase I (2 mg, 0.03 unit) in 0.5 ml buffer activator pH 6.0[1] containing 1 mg tetracosactrin was stirred for 16 h at 37°C then centrifuged. The supernatant was evaporated and portions (10 μl) of the residue (taken up in 100 μl 10% acetic acid) were applied to 20 cm x 20 cm plates precoated with microcrystalline cellulose (Camag, Muttenz) The chromatographic solvent was *n*-butanol–acetic acid–water (60:15:25, by vol.), and peptides were revealed with ninhydrin–cadmium acetate[14]. A comparison of the ability of soluble dipeptidyl aminopeptidase I and Enzacryl Polythiolactone–dipeptidyl amino-peptidase I to degrade tetracosactrin is shown in Fig. 2. Only minor differences can be

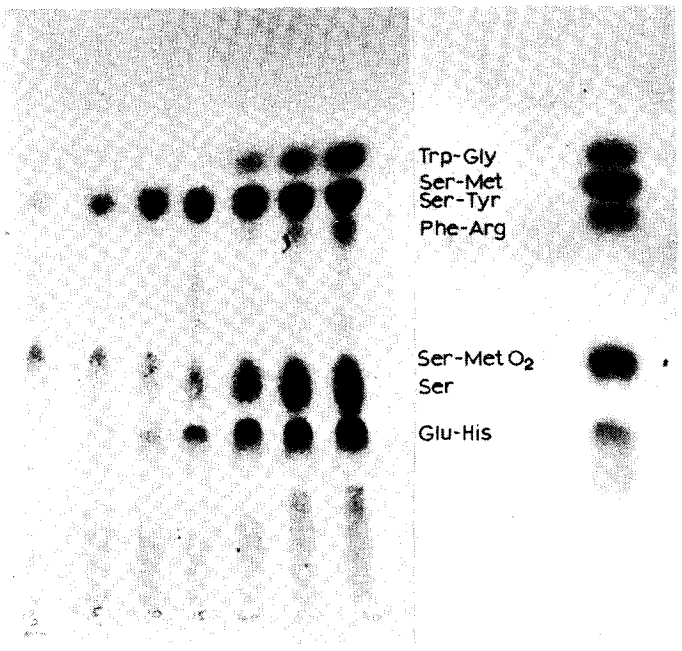

Fig. 2. Degradation of tetracosactrin (β 1–24 ACTH) by soluble and insoluble dipeptidyl aminopeptidase I. Thin-layer chromatography on plate precoated with microcrystalline cellulose. Solvent: *n*-butanol–acetic acid–water (60:15:25, by vol.). Left: progress of hydrolysis with time (1.5–120 min) using soluble enzyme. Right: hydrolysis with Enzacryl Polythiolactone–dipeptidyl aminopeptidase I after 16 h. Detailed conditions in text.

observed between the effects of the two enzyme systems, so that the hydrolytic properties of dipeptidyl aminopeptidase I are largely unaffected by insolubilization. The reaction time (16 h) using insoluble dipeptidyl aminopeptidase I was greater than that (2 h) using native enzyme because of its lower specific activity. Similar results have been obtained with dipeptidyl aminopeptidase I bound to other water-insoluble supports but because of the lower activities of these preparations longer reaction times have been necessary.

A number of problems remain before the full potential of dipeptidyl aminopeptidase I as a sequencing reagent can be realized, of which one is the existence of sequences which are resistant to cleavage. Since such sequences could be cleaved by treatment with other enzymes, *e.g.* trypsin, we reasoned that preparation of dipeptidyl aminopeptidase I covalently bound to water-insoluble polymers would be a valuable step towards the use of a multi-enzyme system in these circumstances. Such a system may have particular value in the investigation and rapid identification of homologous sequences in natural peptides and proteins (haemoglobins, hormones and enzymes from different sources) and for identification of impurities and failure sequences in synthetic peptides.

Little detailed information about the structure of dipeptidyl aminopeptidase I is available beyond the gross arrangement of subunits[10] and there was no reason for

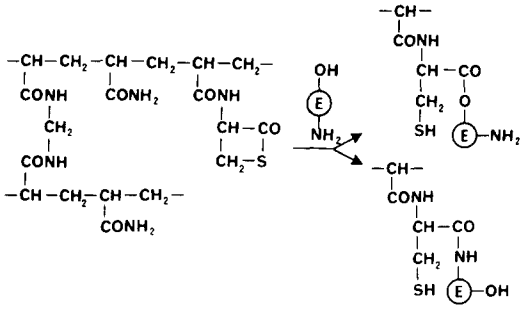

Fig. 3. Structural features of Enzacryl Polythiolactone polymer showing method of attachment of enzyme. E = enzyme.

preferring one polymer or coupling method to any other. In fact, most polymers commercially available for this purpose will couple through the amino groups in the enzyme (Table I), and although insolubilized preparations of dipeptidyl aminopeptidase I were obtained with such supports, their activities were low. However, the acrylamide polymer Enzacryl Polythiolactone possesses several distinctive features. It requires no preliminary activation and coupling can occur under very mild conditions through the hydroxyl groups of serine and tyrosine, as well as amino groups (Fig. 3). Insoluble preparations of dipeptidyl aminopeptidase I using this support showed greater incorporation of enzyme and higher activity than any others. One factor relevant to these observations may be the presence of free thiol groups in the support matrix (Fig. 3), liberated during the coupling procedure. The spatial relationship of these to the enzyme molecules may be of importance because of the thiol requirement of dipeptidyl aminopeptidase I for activity and they might also play a part in stabilizing the enzyme.

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