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The preparation and properties of immobilised dipeptidyl-aminopeptidase I (cathepsin C)

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Dipeptidyl-aminopeptidase I (dipeptidyl-peptide hydrolase, EC 3.4.14.1) from bovine spleen has been immobilized by hydrophobic bonding to alkyl- or aryl-Sepharoses. Optimum binding occurred with octyl- and phenyl-Sepharoses. The activity of the immobilised dipeptidyl-aminopeptidase I has been determined using glycylarginyl-*p*-nitroanilide as substrate and the pH optimum of the immobilised enzyme determined as well as the stability of the enzyme to repeated use. Preliminary studies using immobilised dipeptidyl-aminopeptidase I for the digestion of methione enkephalin have been carried out using reverse-phase HPLC to analyse the reaction.

Introduction

The possibility of using dipeptidyl-aminopeptidase I (dipeptidyl-peptide hydrolase, EC 3.4.14.1) for the determination of the amino-acid sequences at the N-termini of peptides and proteins has been suggested by several authors [1–3]. In connection with our investigations on the use of fast-atom-bombardment mass spectrometry (FAB-MS) in conjunction with dipeptidyl-aminopeptidases I and IV [4], we have been exploring methods for the immobilisation of these enzymes. In this way we hope to obtain stable preparations of dipeptidyl-aminopeptidases I and IV, and the use of immobilised enzymes should simplify the analysis of the N-terminal amino-acid sequences of proteins, as they can readily be removed from the reactions when required before mass spectrometric analysis. We have studied the immobilisation of dipeptidyl-aminopeptidase I by hydrophobic or covalent binding to a polysaccharide support (Sepharose) and now wish to report our findings on the properties of the immobilised enzyme.

Materials and Methods

Dipeptidyl-aminopeptidase I from bovine spleen as a 50% suspension in glycerol was purchased from Boehringer Corporation (London, U.K.) or isolated from bovine spleen by the method of McDonald and co-workers [3]. The 'C_n' series of alkyl agaroses (where n represents the number of carbon atoms in an alkyl chain attached to the agarose) were obtained from Miles Scientific (Slough, U.K.). Octyl- and phenyl-Sepharoses and cyanogen bromide-activated Sepharose were obtained from Pharmacia (Milton

Abbreviations: FAB-MS, fast-atom-bombardment mass spectrometry; Gly-Arg-pNA, glycylarginyl-*p*-nitroanilide.

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Keynes, U.K.). 7-Glycylphenylalaninamido-4-methylcoumarin was purchased from Universal Biologicals (London, U.K.). The acetate salt of glycylarginyl-*p*-nitroanilide (Gly-Arg-pNA) was purchased from Sigma Chemicals (Poole, U.K.).

Hydrophobic immobilisation. A series of columns (6 × 1 ml) of 'C_n'-agaroses ($n = 0$, 2, 4, 6, 8 or 10) were washed with 50 mM potassium phosphate buffer (20 ml (pH 6.7)) [5] which contained 12 mM 2-mercaptoethanol and 10% (w/v) sodium chloride. Dipeptidyl-aminopeptidase I (50 μl 1 U) was added to each column and allowed to interact with the support for 10 min at 17 °C. The columns were then washed with two portions of the same buffer (2 ml) and the combined eluates were tested for unbound enzyme activity, using Gly-Arg-pNA as described below. The activity in the eluate was then determined (Fig. 1). All unbound dipeptidyl-aminopeptidase I was washed from the columns with the 2 × 2 ml portion of buffer described above. Negligible amounts of enzyme were eluated from the columns by further washing.

Enzyme stability. Columns (1 ml) of dipeptidyl-aminopeptidase I, immobilised as described above to phenyl- or octyl-Sepharose, were prepared and the activity of the bound enzyme determined. Repeated enzyme assays using both columns were carried out after 10 min, 1 h, 24 h, 48 h and 96 h (Fig. 2).

Thermal stability. Dipeptidyl-aminopeptidase I (2 U) was immobilised on octyl-Sepharose (8 ml) and the batch divided into eight aliquots of 1 ml. The aliquots were incubated at different temperatures for 10 min, cooled to room temperature (17 °C) and then assayed for retained activity. As a comparison, the soluble enzyme (20 μl of 2 U/ml) was incubated at the same temperatures as above (eight aliquots) and the enzyme activities measured (Fig. 3).

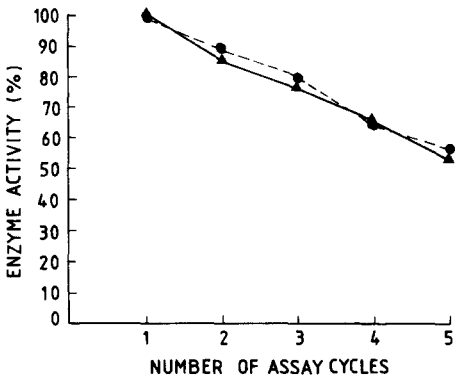

Fig. 2. Effect of a number of assay cycles on the activity of dipeptidyl-aminopeptidase I immobilised on phenyl- or octyl-Sepharose (▲———▲, phenyl-Sepharose; ●·····●, octyl-Sepharose). The assays were performed after 10 min, 1 h, 24 h, 48 h and 96 h; the immobilised enzyme was stored at 4 °C between assays.

Enzyme assays

Soluble dipeptidyl-aminopeptidase I. For each sample of enzyme to be assayed, the substrate Gly-Arg-pNA acetate (10 μl, 9.7 mM) was added in 50 mM potassium phosphate buffer (pH 6.7)

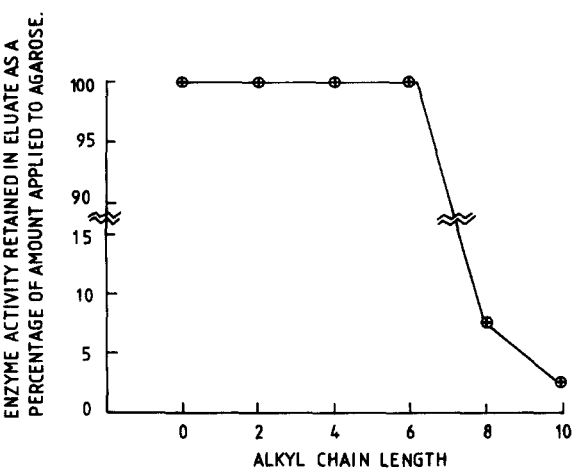

Fig. 1. Variation with chain length of binding of dipeptidyl-aminopeptidase I to alkyl agaroses.

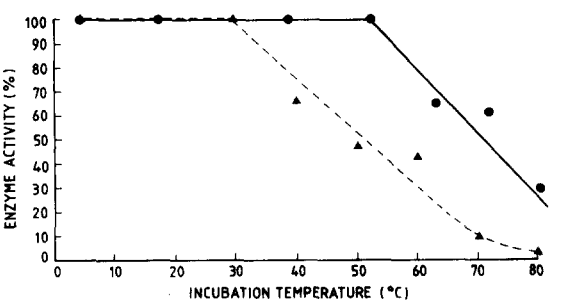

Fig. 3. Thermal stability of dipeptidyl-aminopeptidase I (▲·····▲, immobilised on octyl-Sepharose; ●———●, soluble).

containing 12 mM 2-mercaptoethanol and 10% (w/v) sodium chloride, and the solution was incubated at the required temperature, for approx. 10 min. The absorption at 385 nm was then determined.

Immobilised dipeptidyl-aminopeptidase I. The enzyme immobilised on phenyl- or octyl-Sepharose (1 ml bed vol.) was mixed by gentle inversion two or three times with Gly-Arg-pNA (10 μl, 9.7 mM) in the potassium phosphate buffer solution mentioned above, and the reaction allowed to proceed under the conditions of temperature, time, etc., as required. The immobilised enzyme was washed with a known volume of buffer and the optical absorbance at 385 nm determined. Initial experiments showed that all the *p*-nitroaniline was eluted from the immobilised enzyme under these conditions. The enzyme activation of soluble dipeptidyl-aminopeptidase I preparations were checked using 7-glycylphenylalaninamido-4-methylcoumarin as substrate in place of Gly-Arg-pNA, and the protein concentration was determined by the Hartree modification of the Lowry protein estimation [6].

Digestion of methionine enkephalin by immobilised dipeptidyl-aminopeptidase I. Dipeptidyl-aminopeptidase I [1] (3 U) was immobilised on octyl-Sepharose (2 ml bed vol.) and this was divided into two portions. Each portion was washed in a centrifuge tube with 10 × 1 ml of incubation buffer (50 mM potassium phosphate containing 12 mM 2-mercaptoethanol and 10% (w/v) sodium chloride (pH 5.8)). Methionine enkephalin (40 μl, 15.5 mM) was added to each sample and incubated at room temperature. At different time intervals, the incubations were centrifuged at low speed and an aliquot (01 μl) of the supernatant was subjected to analysis by HPLC (Spherisorb-10 ODS column, 300 × 4.6 mm, elution with 35% acetonitrile/65% H_2O/0.1% trifluoroacetic acid, detection at 214 nm) to study the breakdown of the peptide. After each test, the samples were gently agitated to ensure thorough remixing of the substrate.

Results and Discussion

We have found that dipeptidyl-aminopeptidase I can be immobilised by hydrophobic bonding to octyl- and decyl-Sepharose and also to phenyl-Sepharose, and we have found it convenient to use commercially available octyl-Sepharose as a support for the enzyme. The immobilized enzyme was stable when stored at 4° C, over 50% of the original activity being retained after 4 days and five repeated assays. The rate of hydrolysis of Gly-Arg-pNA by dipeptidyl-aminopeptidase I immobilised on (A) octyl-sepharose and (B) phenyl-Sepharose gave v_0 values of 32% and 37% of the v_0 value obtained for the soluble enzyme under identical conditions. Samples of dipeptidyl-aminopeptidase I immobilised on octyl- or phenyl-Sepharose showed a slight drop in activity (approx. 20%) of enzyme activity after 24 h storage before use. After this time, no change in enzyme activity was observed after storage for up to 14 days before use. Repeated use of the same sample of immobilised dipeptidyl-aminopeptidase I caused a small regular loss in enzyme activity with each assay, as shown in Fig. 2.

The enzyme immobilised on octyl-Sepharose was capable of digesting Met-enkephalin and after 30 min, at room temperature, Tyr-Gly and the tripeptide Gly-Phe-Met could be detected by HPLC. After a further 50 min degradation of the tripeptide to Gly-Phe and Met could be seen. After 200 min of digestion, the Met-enkephalin peak had disappeared completely. The rate of digestion of Met-enkephalin by the immobilised dipeptidyl-aminopeptidase I was approx. 20% the rate of digestion by the soluble enzyme under comparable conditions and, thus, the immobilised enzyme should be of use for the slow, controlled digestion of peptides and proteins for sequential analysis by mass spectrometry.

The attachment of enzymes to hydrophobic polysaccharides is an established technique for the immobilisation of a range of enzymes under mild conditions [7,8,9]. We have found that immobilisation of dipeptidyl-aminopeptidase I by hydrophobic bonding to commercially available octyl- or phenyl-Sepharose was far superior to immobilisation on cyanogen bromide-activated-Sepharose [10]. Dipeptidyl-aminopeptidase I immobilised by this latter method was inactive, possibly because the pH used for immobilisation was too high for dipeptidyl-aminopeptidase I to remain stable for any length of time. However, reduction

of the pH of the immobilisation reaction to a pH value of 7.5 did not lead to the production of active immobilised enzyme. Alternatively, the covalent bonding may also change the secondary and tertiary structure of this multi-subunit enzyme, which may have altered the activity of the enzyme.

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