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C-terminal labelling of β-casein

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This paper is the first to report specific labelling of a native protein at its C-terminal end by carboxypeptidase Y-catalyzed transpeptidation between β-casein and tritiated Phe amide. A tryptic digest of the radiolabelled protein was resolved by reversed-phase HPLC and a single labelled peptide was isolated therefrom. Sequence determination and FAB mass spectrometry showed that the last 2 residues (Val-209, Ile-208) of β-casein had been deleted and Ile 207 substituted by Phe, deamidation presumably occurring after transpeptidation. Identical results were obtained by transpeptidating the isolated C-terminal tryptic heptapeptide (203–209) of native β-casein.

β-Casein; C-terminal peptide; Protein labeling; Carboxypeptidase Y; Transpeptidation

1. INTRODUCTION

Specific labelling at the C-terminus would lead to significant advances in protein biochemistry. Such a method could be useful for metabolic studies and for the isolation of the C-terminal peptide in a protein hydrolysate.

As far as we know, there is no chemical method for specific labelling of the terminal carboxyl group without partial labelling of the side carboxyl functions. It has been shown that, under suitable conditions, CPD-Y, a serine exopeptidase, could catalyze the substitution of the C-terminal amino acid by an exogenous amino acid or amino acid amide (transpeptidation) [1]. Although the mechanism of such a reaction is not fully understood, it seems obvious that the accessibility of a protein at its C-terminus to CPD-Y is an absolute necessity. As a model we chose bovine β-casein because the amino acid sequence of this protein is known and its C-terminus is quite accessible to CPD-A [2].

To our knowledge this is the first report of the transpeptidation of a protein.

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Abbreviations: CPD-Y, yeast carboxypeptidase; Phe-NH_2, Phe amide; [^{3}H]Phe-NH_2, tritiated Phe amide; RP-HPLC, reversed-phase high performance liquid chromatography; CPD-A, carboxypeptidase A; DFP, diisopropylfluorophosphate; TFA, trifluoroacetic acid; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; PITC, phenylisothiocyanate; PTH, phenylthiohydantoin

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Lichrosolv acetonitrile was from Merck; TFA from Pierce; DFP and cold Phe-NH_2 from Sigma; TPCK-treated trypsin (155.9 U/mg) and CPD-Y (100 U/mg) were from Worthington and Merck, respectively. Other chemicals were of reagent grade. [^{3}H]Phe-NH_2, with a specific radioactivity of 56 Ci/mmol, was prepared as described [3].

2.2. *Preparation of substrates*

β-Casein A^2 was prepared as described in [4]. Its C-terminal heptapeptide was purified by RP-HPLC from a tryptic hydrolysate. The purity of

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the peptide was assessed and its concentration determined from amino acid analysis. Tryptic digestions were performed at 30°C for 6 h in 100 mM NH_4HCO_3, pH 8.5, with TPCK-treated trypsin (1:100, w/w, with respect to substrate).

2.3. *Hydrolysis by CPD-Y*

Substrates were digested at 37°C in 100 mM Na phosphate, 100 mM KCl, 1 mM EDTA buffer, pH 7.5, with $E/S = 1:100$. Aliquots were taken at intervals and the reaction was stopped by adding DFP (0.1 mM final). They were dried and determination of the hydrolysis state of the substrate was performed as follows.

When β-casein was used as a substrate, 200 μl of 100 mM citrate buffer, pH 2.2, was added to each dried aliquot. After centrifugation and filtration, the supernatant was injected onto the amino acid analyzer.

When the C-terminal heptapeptide was used as a substrate, each aliquot was separated by RP-HPLC. Elution conditions allowed an excellent separation and recovery of all the peptide species, which were identified by amino acid analysis. Quantification was performed from the chromatogram by using a linear calibration curve obtained by plotting peak heights against known injected amounts of each peptide.

2.4. *Transpeptidation reactions*

They were performed at 37°C in 100 mM Na phosphate, 100 mM KCl, 1 mM EDTA buffer, pH 7.5. E/S was 1:100 and the substrate/Phe-NH_2 ratio was 1:10 (mol/mol). The action of CPD-Y was stopped by adding DFP (0.1 mM final). Identification and analysis of the reaction products are described in the text for β-casein. For the peptide, the reaction products were separated and quantified as previously described for the hydrolysis reaction.

2.5. *RP-HPLC*

The equipment consisted of a 720 system controller, two 6000 A pumps, a WISP, a data module and a 441 detector (214 nm) from Waters. A Waters μBondapak C18 column was used with a flow rate of 1 ml/min at 40°C.

2.6. *Amino acid analyses*

They were carried out on a LC 5000 amino acid analyzer (Biotronik), or by RP-HPLC according to [5] after conventional acid hydrolysis and PITC derivatization.

2.7. *Sequencing*

Manual Edman degradations were performed according to [6]. PTH derivatives were separated on a Nova-Pak C18 column (Waters) according to S. Cohen (Waters, personal communication).

2.8. *FAB mass spectrometry*

A Kratos MS80 spectrometer with energy of the xenon atoms adjusted to 7 kV was used.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of hydrolysis of β-casein by CPD-Y*

The rate of release of the C-terminal residues was extremely high. Under the conditions described in section 2, complete liberation of Ile and Val was observed after 20 s. This demonstrates the accessibility of the C-terminal part of β-casein to CPD-Y.

3.2. *Transpeptidation of β-casein by [^{3}H]Phe-NH_2*

After incubating the protein for 2 h in the presence of CPD-Y and [^{3}H]Phe-NH_2 as previously described, a first dialysis against a solution of cold Phe-NH_2 (1 mM) in Na phosphate buffer (10 mM, pH 7.5) followed by 2 dialyses against Na phosphate buffer alone were performed to remove the Phe-NH_2 which could be non-covalently bound to the protein.

A significant amount of incorporation of radioactivity was observed in the CPD-Y-treated sample compared to the protein incubated in the same conditions without enzyme (570000 cpm and 21500 cpm for 2 nmol of β-casein incubated with and without CPD-Y, respectively). To determine the location of the label, the radioactive casein was chromatographed on RP-HPLC with a 10 mM Na phosphate buffer, pH 7.0/CH_3CN system. Fractions (1 ml) were collected during the run and radioactivity was counted on aliquots (10 μl) of all fractions. Radioactivity was clearly associated with the protein peak. The pooled radioactive fractions were dried and the protein was subjected to a tryptic hydrolysis (cf. section 2). The hydrolysate was separated by RP-HPLC (fig.1). The radioactivity

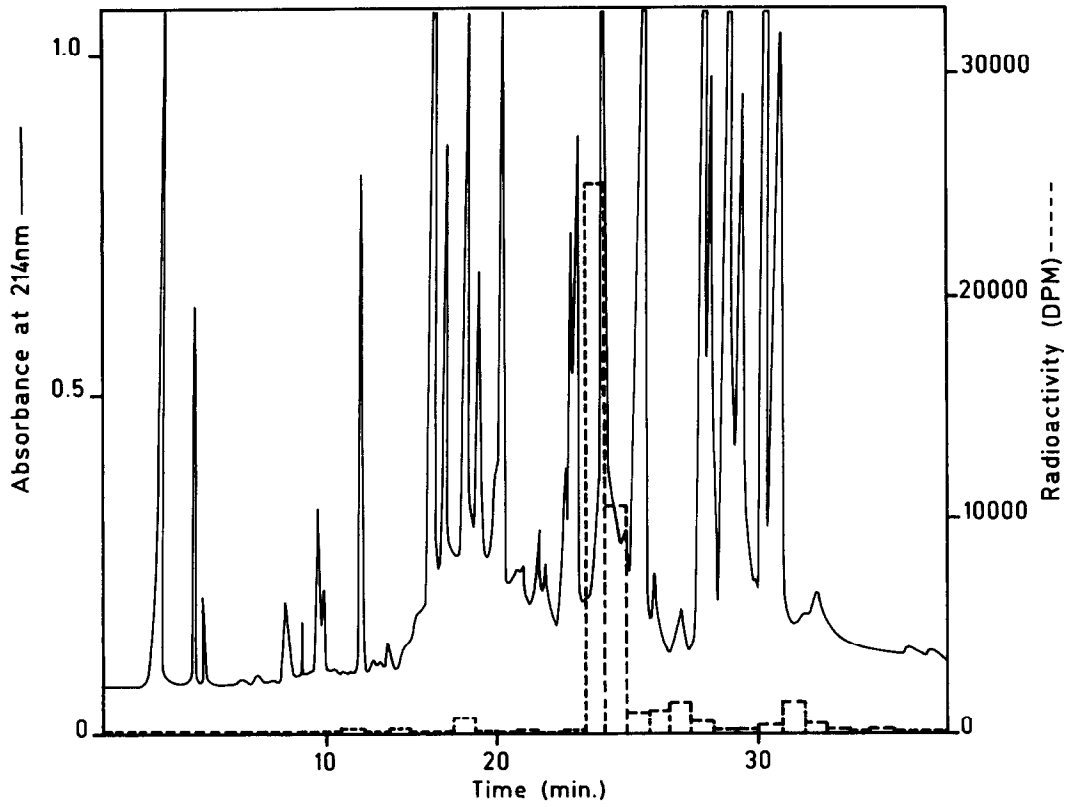
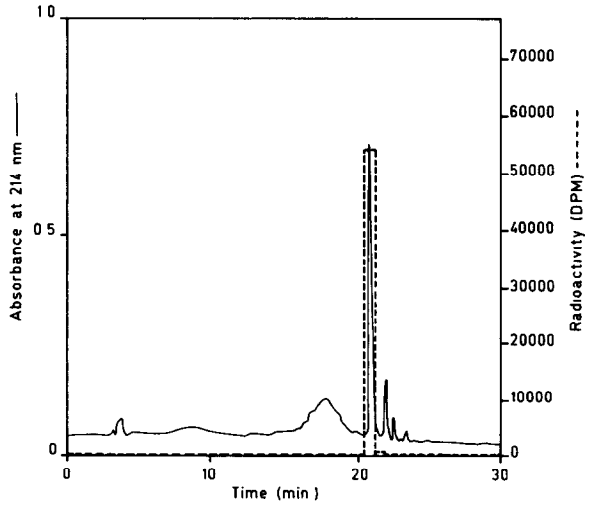


Fig.1. Separation by HPLC of a tryptic hydrolysate of transpeptidated β-casein. Linear gradient from 100% solvent A (0.12% TFA) to 100% solvent B (75% CH_3CN/25% H_2O/0.10% TFA final); fractions of 1 ml; radioactivity measured on 10 μl of each with a LS 1800 scintillation counter (Beckman).



was located in only one part of the chromatogram. The corresponding fraction was rechromatographed in another solvent system, giving a single radioactive peak (fig.2). The sequence of the corresponding peptide was: G-P-F-P-F, while that of the original C-terminal tryptic heptapeptide (203–209) of bovine β-casein was G-P-F-P-I-I-V. Thus, transpeptidation had occurred at position 207, Ile being replaced by [^{3}H]Phe-NH_2.

The peptide G-P-F-P-F was analyzed by FAB mass spectrometry. The result (MH^+ = 564) con-

Fig.2. HPLC rechromatography of the radioactive fraction from fig.1 in another solvent. Linear gradient from 100% solvent A (25 mM ammonium acetate, pH 6.2) to 100% solvent B (60% CH_3CN/40% 50 mM ammonium acetate, pH 6.2). Fractions of 0.5 ml; radioactivity measured on 10 μl of each.

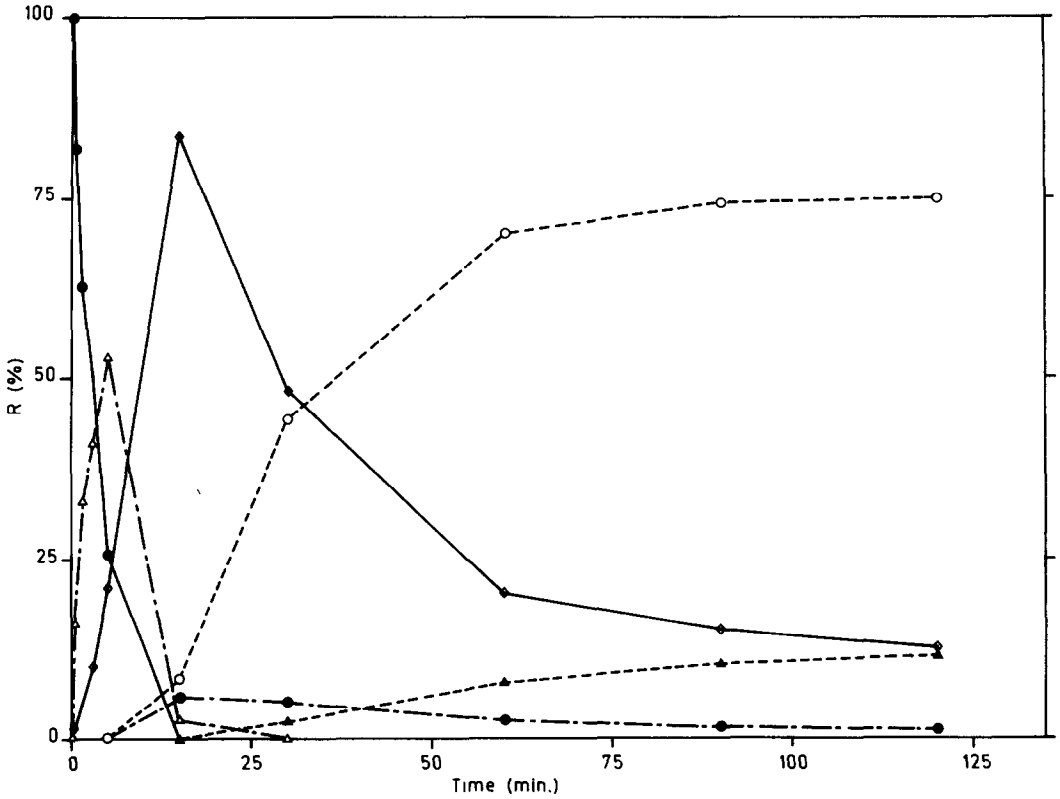


Fig.3. Kinetics of transpeptidation of the C-terminal heptapeptide of bovine β-casein by CPD-Y. See section 2 for details of the reaction conditions. R (%), relative molar proportion of each molecular species as a function of time. (●——●) G-P-F-P-I-I-V; (△—·—△) G-P-F-P-I-I; (◇——◇) G-P-F-P-I; (○···○) G-P-F-P-F; (●—·—●) G-P-F-P-FNH_2; (▲···▲) G-P-F-P.

firmed the sequence previously determined but indicated that the C-terminal function was a carboxylic one and not an amide. Since we did not observe any deamidation of free Phe-NH_2 by CPD-Y under the same conditions in another experiment, it is very likely that Ile-207 had been replaced by Phe-NH_2 and that deamidation had occurred afterwards.

A transpeptidation reaction was performed under the same experimental conditions on the C-terminal heptapeptide of β-casein purified as described in section 2. As with the entire protein, removal of Val-209 and Ile-208 took place, and then transpeptidation on Ile-207 occurred. Moreover, at 120 min reaction time (same as for β-casein) the C-terminal Phe of the transpeptidated peptide was deamidated at almost 100% (fig.3).

The yield of transpeptidation for the peptide was ~75% and slightly above 60% for the protein. The similarity of these figures suggests that the site of interaction between CPD-Y and its substrate accommodates only a few amino acid residues. This was confirmed by the hydrolysis kinetics of the C-terminal heptapeptide by CPD-Y (not shown) which was extremely close to that obtained for the native protein.

We wondered whether the transpeptidation position (Ile-207 in our case) could be modified by changing the reaction conditions. As the results were identical irrespective of the substrate (protein or C-terminal peptide), all further experiments were performed on the C-terminal heptapeptide. The ratios E/S, Phe-NH_2/S, the ionic strength and the water content were changed in different experiments (only one parameter being changed each time). In all the cases, transpeptidation occurred at Ile-207 (with, of course, different yields according to the experimental conditions). No substitution of

residues Ile-208 or Val-209 was observed. Thus, it seems that the amino acid sequence itself is responsible for the transpeptidation of Ile-207 only. It is clear that a Pro in position $(n-1)$ is not an absolute requirement since a few examples of transpeptidation of short peptides by Phe-NH_2 in other positions have been reported: gastrin-like peptides (caerulein, C-terminal octapeptide of cholecystokinin and pentagastrin) have been transpeptidated at their original C-terminal Phe-NH_2 by [^{3}H]Phe-NH_2, an Asp being located at position $(n-1)$ [7]. Thus, it can be suggested that the slow hydrolysis of Pro-X or Asp-X bonds could be a favorable situation.

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