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Monitoring tripeptidase activity using capillary electrophoresis

Comparison with the ninhydrin assay

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ABSTRACT

Capillary electrophoresis (CE) was used to assay the activity of a tripeptidase from a crude extract of *Lactococcus lactis* subsp. *lactis* NCDO 712 against the substrate, Gly–Gly–Phe and a comparison with a standard ninhydrin assay was made. Standard curves of the substrates and products showed a significantly variable colorimetric reaction to ninhydrin making accurate quantification of the tripeptidase problematic. The CE assay further demonstrated that the presence of contaminating enzymes in crude cell-free extracts can cause secondary reactions that are not apparent from the ninhydrin assay data. The CE assay was also able to generate enzyme kinetics data and monitor, during purification, the presence of co-eluting contaminating activities. The speed and sensitivity with CE allows routine analysis of the tripeptidase activity without any derivatization normally required for this enzyme.

INTRODUCTION

Enzymology has frequently used chromogenic or fluorogenic substrates to assay for enzyme activity during purification procedures and also to characterize enzymes kinetically. This is particularly true for peptidolytic enzymes such as aminopeptidase N (EC 3.4.11.2) and dipeptidyl peptidase IV (EC 3.4.14.5), which commonly use p-nitroanilide (pNA) derivatives of amino acids and peptides such as Lys–pNA [1] and Gly-Pro-pNA [2], respectively, to assay for activity. Some other proteolytic enzymes, however, are not able to use these type of substrates and require other methods of assay. One such enzyme is tripeptidase (EC 3.4.11.4) which cleaves amino acids from the N-terminal of tripeptides. This enzyme has now been purified from a number of sources, with several different methods used to assay for activity; by the increase in free amino groups detected colorimetrically by reaction with ninhydrin and also by an amino acid oxidase coupled reaction [3]; and by amino acid analysis of the enzyme reaction products [4].

These methods of analysis can be problematic. Amino acid analysis can be slow, requiring long run times with reconditioning of the column following each analysis. The ninhydrin assay, whilst being the most rapid, is subject to an inherent background from the substrate, resulting in problems measuring low enzyme activity, and the different amino acids and peptides are subject to a significantly variable colorimetric reaction with the ninhydrin. The coupled assay methods restrict the measurement of activity to substrates whose components following hydrolysis are able to be quantified in the second coupled reaction. Furthermore, due to the time factor, more detailed substrate specificities are normally only measured qualitatively using thin-layer chromatography and ninhydrin spot detection [5].

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To overcome some of these problems, capillary electrophoresis (CE), ideally suited to monitoring small peptides, was invetigated to see if it could be applied to tripeptidase analysis and compared to ninhydrin assay.

MATERIALS AND METHODS

Organism and preparation of cell extracts

Lactococcus lactis subsp. ***lactis*** NCDO 712 [obtained from the National Collection of Dairy Organisms (now the National Collection of Food Bacteria, NCFB), Earley Gate, Reading, UK] was grown in 1.5-1 batch cultures of Ml7 broth (Difco, East Molesey, UK) containing 5 g lactose l^{-1}, incubating overnight at 30°C prior to harvesting. Cultures were centrifuged at 1000 g for 30 min at 4°C and washed twice in 50 m*M* Tris–HCl buffer, pH 7.5. Cells were disrupted by four passes through a French Pressure Cell at 100 MPa and the debris removed by centrifugation at 20 000 g for 30 min to give the cell free extract.

Assay of tripeptidase and dipeptidase activity

20 m*M* Gly-Gly-Phe or Gly-Phe (Sigma, Poole, UK) (100 μl), water (60 μl) and 10 m*M* sodium phosphate buffer, pH 7.5 (200 μl) were mixed in microfuge tubes (Scotlab, Bellshill, UK). Crude cell extract [or 50 m*M* Tris–HCl buffer, pH 7.5 for blank] (40 μl) was finally added and the tubes incubated at 37°C. The reaction was stopped by the addition of 0.1 *M* acetic acid (100 μl). For a zero-time assay the stopping reagent was added prior to the addition of the crude cell extract. The samples were then centrifuged at 10 000 g for 5 min before assaying by either the ninhydrin or the CE method.

Ninhydrin assay

Method 2 of Doi *et al.* [6] was used. Assay samples (0.2 ml), diluted tenfold with 0.1 *M* acetic acid, were mixed with freshly prepared ninhydrin reagent (0.4 ml) and heated in a water bath at 100°C for 5 min. The tubes were then cooled in an ice-water bath and diluted with acetone (0.3 ml) followed by water-O.1 *M* trisodium phosphate [1:6 (v/v); 0.7 ml]. Absorbance was then measured at 570 nm on a DU7000 spectrophotometer (Beckman, High Wycombe, UK).

Capillary electrophoresis running conditions

Samples were assayed on a Model 270A capillary electrophoresis apparatus (Applied Biosystems, Warrington, UK). Following sample injection (1.5 s by vacuum), running conditions were 30 kV at 45°C for 11 min on a standard 72-cm capillary in 20 m*M* sodium citrate buffer, pH 2.5 (Applied Biosystems). Electrophoretic mobility was monitored by measuring the absorbance at 200 nm. The data was collected at 2 Hz and analyzed using a Drew Roseate Chromatography Data Analysis Package (Drew Scientific, London, UK), with a 100-mV input using external standards calibration for Phe, Gly-Phe and Gly–Gly–Phe.

Preparation of standard curves

Gly, Phe, Gly-Phe and Gly-Gly-Phe standards (Sigma) were prepared from 20 m*M* stock solutions by diluting with water over the range 0.2-1.0 m*M* (0.1–0.5 μmol in assay volume) for the CE analysis and 0.04–0.2 m*M* (0.008-0.04 μmol in assay volume) for the ninhydrin assay. Standard conditions of analysis for CE and ninhydrin assays were then applied.

Zon-exchange chromatography

Cell-free extract (20 ml) was applied to a 20 × 3.2 cm I.D. Q-Sepharose Fast Flow Column (Pharmacia, Milton Keynes, UK) and a 2-l linear gradient of O-O.5 *M* NaCl in 50 m*M* Tris–HCl, pH 7.5 applied at 2 ml/min collecting 4-ml fractions.

Protein determination

Protein concentrations were determined by the method of Bradford [7].

Unless otherwise stated, all chemicals were from BDH, Poole, UK.

RESULTS AND DISCUSSION

In enzymology the selection of substrate is crucial in establishing a suitable assay for the enzyme under investigation. Gly-Gly-Phe was chosen for the lactococcal tripeptidase as this peptide would be unlikely to react with any other enzyme from this source. The lactococcal aminopeptidases are reported not to be active against this peptide or N-terminal glycine residues [1,8,9]. As no carboxypeptidase activity is found in lactococci [10], the prod-

ucts of the tripeptidase reaction on Gly–Gly–Phe are Gly and Gly-Phe. No Gly-Gly can be formed and Phe will only be observed due to breakdown of the Gly-Phe by a dipeptidase. For the CE assay, the inclusion of Phe, with its aromatic ring, increases the absorbency of the peptide to UV monitoring. It also allows detection of any subsequent secondary reaction by the dipeptidase found in lactococci by monitoring the formation of free phenylalanine.

The variable response of the ninhydrin reaction to different amino acids and peptides is well documented [6]. The proposed lactococcal tripeptidase assay can have up to four ninhydrin-positive components present, which may have a variable colorimetric response to the ninhydrin reaction. Fig. 1 shows the variability obtained when using these components to prepare standard curves. Whilst none of the curves were identical, Phe in particular gave a significantly different colorimetric response to the others. Using a single standard curve for quantifying the increase in ninhydrin-positive material formed during a reaction is, therefore, not a reliable method of assaying tripeptidase activity. The only way of quantitatively assaying systems involving multiple ninhydrin-positive components would be to incorporate some separation procedure such as HPLC or CE prior to quantification.

Calibration of the capillary electrophoresis system

Whilst the ninhydrin derivatization does allow detection of UV-invisible amino acids such as Gly, and increases the detectability of UV-visible components such as Phe, the level of sensitivity possible by this technique is not necessarily required in an enzymatic assay. A technique able to accurately detect less than a μmole of a component of interest is usually sufficient for an enzyme assay. The direct measurement of the UV-absorbing Phe-containing peptides at 200 nm on CE showed that this was possible. External standards of the substrate and the known UV-absorbing products of the tripeptidase and dipeptidase reactions were prepared and run on the CE. Table I shows the consistent separation obtained by CE for these components, and the good correlation between the peak area and amount of each standard over the range 0–0.5 μmol/500 μl assay volume, thus allowing accurate determination of the amount of peptide to be found in an unknown sample.

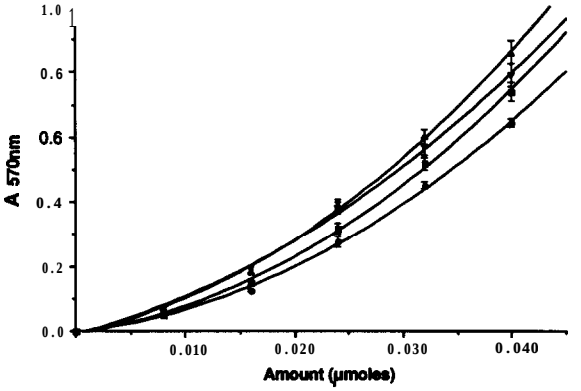

Fig. 1. Ninhydrin reaction standard curves. ■ = Gly; ● = Phe; A = Gly-Phe; ▼ = Gly–Gly–Phe. Error bars indicate 2S.D. (*n* = 14).

Tripeptidase assay

To determine the effectiveness of the two techniques, crude lactococcal extract was assayed for tripeptidase activity by both the ninhydrin assay and the CE method. Fig. 2 demonstrates that whilst a linear increase in absorbance over time is found with the ninhydrin assay, reflecting an increased amount of the ninhydrin positive material and suggesting a simple reaction with single product formation, the CE reveals that the reaction is more complex than the formation of a single product with Phe being produced by a contaminating dipeptidase activity. Using the ninhydrin assay during a purification to monitor the specific activity value is therefore prone to an error which will be variable, dependant on the amount of contaminating enzymes present. The CE assay by measuring the individual components is able to take this into account.

Kinetic analysis using CE assay

The observation from Fig. 1 that the products formed have a variable colorimetric response to ninhydrin means that accurate kinetic analysis, even after purification of the tripeptidase from enzymes reponsible for the secondary reactions, can only be achieved by first separating the products prior to quantification. Using purified enzyme, the tripeptidase CE assay was repeated varying the Gly–Gly–Phe concentration (2.5–10 m*M* final concentration) and incubating for 10 min. Fig. 3 shows

TABLE I

ELECTROPHORETIC MOBILITY AND LINEAR REGRESSION ANALYSIS OF TRIPEPTIDASE ASSAY STANDARDS BY CE

Component	Mean electrophoretic mobility (min)	Standard deviation (min)	Linear regression analysis[a] (y =)	Linear regression coefficient
Gly–Gly–Phe	5.966	0.013	416.48x + 1.7802	0.995
Gly-Phe	5.684	0.018	316.15x + 0.7983	0.999
Phe	9.876	0.058	440.67x + 4.9198	0.998

[a] y = Peak area from data analysis; x = amount of component injected over the range O-0.5 μmol/500 μl assay volume.

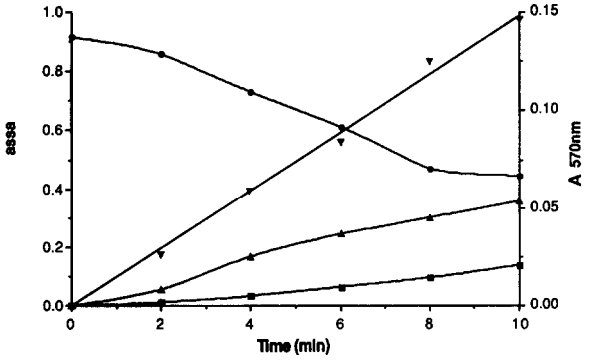

Fig. 2. Tripeptidase reaction monitored over time using the ninhydrin assay and the CE. ▼ = Ninhydrin reaction (y = $-$ 1.8095 10^{-3} + 1.5029 $10^{-2}x$, r^2 = 0.995; ● = Gly–Gly–Phe; A = Gly-Phe; ■ = Phe.

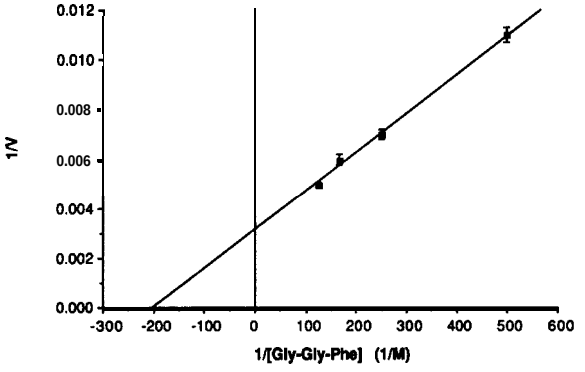

Fig. 3. Lineweaver-Burk plot of tripeptidase activity against Gly–Gly–Phe. V = μmol Gly-Phe formed/(min · mg protein); y = 3.1795 10^{-3} + 1.5631 $10^{-5}x$, r^2 = 0.997; V_{max} = 63975 μmol/(min mg protein); K_m = 4.91 mM. The error bars indicate 2S.D. (n = 4).

the resulting Lineweaver-Burk Plot obtained, with a K_m of 4.91 mM for the substrate and a V_{max} of 63 975 μmol Gly-Phe formed/(min · mg protein) [11].

Other benefits of the CE assay

The separation of the products prior to quantification also allows a clear discrimination between different peptidase activities to be made, even if the enzymes co-eluted during a purification step. This is demonstrated in Figs. 4 and 5, where Gly-Gly-Phe

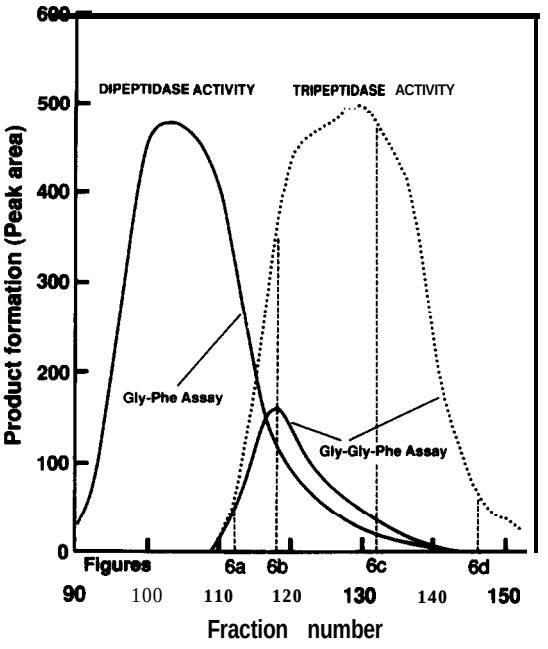

Fig. 4. Q-Sepharose fast flow chromatography monitoring dipeptidase and tripeptidase activities by CE. — = Phe formation; = Gly-Phe formation.

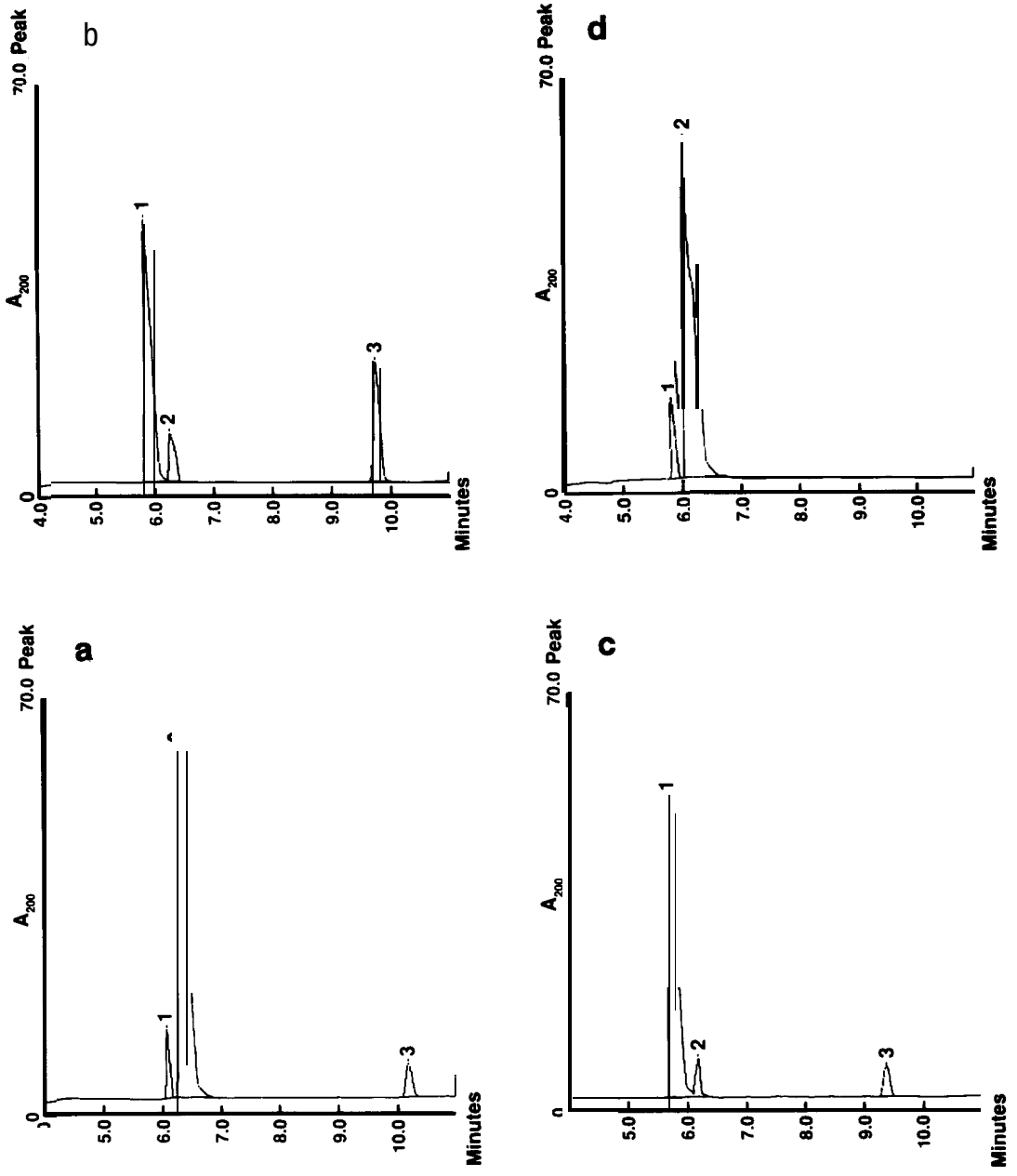

Fig. 5. CE electropherograms of CE assay using Gly–Gly–Phe, monitoring eluent from Q-Sepharose fast flow column. Peaks: 1 = Gly-Phe; 2 = Gly–Gly–Phe; 3 = Phe. (a) Fraction 112; (b) fraction 118; (c) fraction 132; (d) fraction 146.

was used to analyse the elution of tripeptidase and coeluting dipeptidase from Q-Sepharose Fast Flow column. The electropherograms (Figs. 5a-d) show that as elution progressed the ratios of different products formed changes, indicating that more than one activity was responsible for the observed activity. To confirm this the fractions where assayed with Gly-Phe as substrate. Fig. 4 shows the presence of the earlier eluting dipeptidase activity followed by the tripeptidase activity. This would assist

during a purification, showing which fractions should be pooled to prevent contaminating dipeptidase activity being present in future purification steps.

CONCLUSIONS

CE has been shown to be an effective method of assaying tripeptidase activity compared to the ninhydrin assay commonly used for this enzyme. It is able to produce kinetic data that is unambiguous, relating directly to either specific product formation or substrate hydrolysis. The technique also readily allows discrimination between co-eluting activities.

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