FLUORESCENCE APPLICATIONS

LUMINESCENCE ANALYSIS OF THE ACTION OF TRYPsin ON CASEIN USING THE MODEL LS-50

ABSTRACT

Casein, a phosphoprotein consisting of 199 amino acid residues, is hydrolyzed by a variety of proteases, reflecting the physiological role of the protein. The hydrolysis characteristics of casein make it an ideal model for many proteolytic enzymes, (1,2) typical applications of this are in the pharmaceutical industry where the effect of novel drugs on digestive processes can be evaluated, and in clinical biochemistry where the activities of specific enzymes are investigated.

INTRODUCTION

Two aspects of the digestion of casein by proteases have been investigated using the PerkinElmer Model LS-50 Luminescence Spectrometer, these being (i) the kinetics of digestion, carried out using the Model LS-50 fitted with the 4-position stirred cell changer (Part No. L225-0134) and (ii) the effect of digestion on the electrophoretic mobility of casein and its breakdown products using the plate reader accessory (Part No. L225-0137).

In both cases analysis was performed on fluorescein isothiocyanate labeled casein. This labeling provides two major benefits: firstly the labeling ratio of approximately 4:1 produces a high degree of self-quenching of fluorescence in the intact protein. When casein is digested to smaller fragments, the quenching effect is removed, producing an increase in fluorescence dependent on the rate of digestion. This enables measurement of protease kinetics simply by monitoring the fluorescence intensity of the suspension. Secondly, the fluorescein label can be monitored after electrophoresis of breakdown products, to provide fluorescence microdensitometergrams of various protease digestion products.

MATERIALS

Casein FITC conjugate type I I (C-3777), trypsin type II (T8128), protease type X (P1512) and sodium dihydrogen orthophosphate (S0751) were obtained from Sigma Chemical Company Limited, Poole UK. Disodium hydrogen orthophosphate (AnalaR grade) and sodium chloride (AnalaR grade) were obtained from BDH Chemicals.

Agarose (electrophoresis grade) was obtained from Bio-Rad.

Deionized water was used throughout.

Casein stock suspension was prepared by the addition of 39 mg of casein-FITC to 10 mL of 0.1 N phosphate buffer, pH 7.4.

Trypsin and protease stock suspensions were prepared by suspension of 11 mg of protein in 10 mL of 0.1 N phosphate buffer, pH 7.4.

METHODS

Proteolytic digestion of casein-FITC

Digestion of casein by trypsin or protease was carried out by incubation of 1.0 mL of casein stock suspension with either 5 µL of trypsin stock suspension or 10 µL of protease stock suspension. Digestion was stopped by rapid cooling to 4 °C.
Agarose electrophoresis of casein

3% agarose gels were prepared and cast according to the Bio-Rad Applications Sheet (3). Electrophoresis was carried out using the Bio-Rad miniblot system. Gels were loaded with 10 µL of sample for each lane and run at 100 volts for 90 minutes. Scanning was carried out using the plate reader accessory.

Casein digestion kinetics

20 µL of trypsin stock suspension was added to each of 4 cuvettes containing 2 mL of casein stock suspension and the wavelength program routine used to collect constant interval data. Samples were thermostatted at 37 °C and stirred using the 4 position thermostatted stirred cell changer accessory L225-0134.

RESULTS

Time dependent data for the tryptic digestion of casein were collected for 4 different substrate concentrations and the initial velocities of each were calculated and inserted into the Enzfitter* enzyme kinetics routines. Figure 1 shows the initial velocity data for casein digestion by trypsin with (inset) the Lineweaver-Burke and Eadie-Hofstee plots.

The effect of digestion of casein by trypsin and protease on electrophoretic mobility is shown in Figure 2. The intact protein shows several distinct bands of relatively low mobility. Proteolytic digestion causes the appearance of distinct high mobility bands in the case of trypsin and a broad, featureless band for protease. Differences in the mobility patterns caused by the two proteolytic enzymes are due to the enzyme specificity. Trypsin hydrolyzes proteins wherever accessible lysine or arginine residues are found and thus will produce a predictable number of fragments having relatively small variation in molecular weight. Protease, consisting of a range of non-specific proteolytic enzymes, will cleave many more sites producing a large number of fragments of very varied molecular weight.

Calibration data for casein-FITC electrophoresis was obtained by running a single gel with 4 different concentrations of casein-FITC. Results obtained were used to prepare the calibration graph for casein-FITC on electrophoresis gels (Figure 3).
CONCLUSIONS

Enzyme kinetics analysis and fluorescence microdensitometry of electrophoresis gels can be routinely performed using the PerkinElmer Model LS-50 Luminescence Spectrometer and accessories.

Kinetic data can be collected simultaneously for 4 separate cuvettes, then stored in a format directly compatible with Enzfitter where the data can be manipulated using Michaelis-Menten, Lineweaver-Burke and Eadie-Hofstee treatments.

Electrophoresis gels can be scanned to provide qualitative and quantitative information on proteins and biological macromolecules. Sample media can also include blotting materials, TLC plates and polyacrylamide gels up to 8x10 cm in size.
REFERENCES

3. DNA Electrophoresis Cell Instruction Manual, Bio-Rad Laboratories

*Enzfitter is a product of Elsevier-Biosoft