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Detection of enzyme activity in fractions collected from free solution capillary electrophoresis of complex samples

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ABSTRACT

Crude fermentation broth from a fermentation of *Aspergillus oryzae* was analyzed using free solution capillary electrophoresis (FSCE) in a alkaline running buffer. Fractions as large as possible were collected after FSCE separation and analyzed for alkaline protease activity with Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide as substrate. Two peaks were isolated; one of them was unknown and therefore was further investigated. After amplification of the activity by incubation with Suc Ala Ala Pro Phe *p*-nitroanilide or casein as substrate, the reaction mixtures were analyzed by FSCE. In this way as little as 3 ng of enzyme were identified as an alkaline protease of the subtilisin family.

INTRODUCTION

For a long time we have been trying to analyze complex fermentation broth for secreted proteins and primary and secondary metabolites. The usual techniques have been thin-layer chromatography for the metabolites and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with Western blotting, overlayer or direct staining technique for the proteins, all fairly time-consuming, laborious and semiquantitative methods.

Aspergillus oryzae is a filamentous fungius with a remarkable ability to secrete a large amount of different enzymatic activities [1]. We have become more and more interested in this organism as a host for "heterologous protein" products, such as the new lipase from *Humicola insolens* [1], found to be a new, very efficient remover of fat stains on cloth. In this context the proteolytic enzymes produced by the native host are undesirable and we therefore wanted to investigate some of the most important of these enzymes, and to be able to detect them when changing the fermentation process. We regard the alkaline proteases as the most aggressive enzymes for "heterologous protein" production and therefore first concentrated on the detection of these.

Free solution capillary electrophoresis (FCSE) is a new analysis technique anticipated to have a high resolution of charged components with a high and efficient sample capacity and therefore probably well suited for solving our problem.

We therefore tried to separate the complex *A. oryzae* fermentation broth into as many components as possible using FSCE and to identify and quantify some of the

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proteolytic enzymes in the fractions. The fractions collected by FSCE were very small, typically of the order of nanograms. Normally the electrical circuit has to be completed through the collection, which gives a high dilution, because the electrode needs to be in the buffer. It is possible to collect samples without dilution, using a capillary with an on-column frit structure, grounding the capillary on its side prior to its outlet [2]. However, normally the fractions are collected in 5–10 μl of buffer. By collecting the same fraction from several runs, higher concentrations are achieved [2–5]. The chance of successfully identifying and quantifying small amounts of enzyme can be increased by reacting the enzyme over many hours with a selected substrate.

In spite of these facts, fractions collected from high-performance capillary electrophoresis (HPCE) have been used for microsequencing on high-performance liquid chromatography (HPLC) [6,7], slab-gel electrophoresis (labeled with radioactivity [8]), determination of amino acid composition using HPLC [4], mass spectrometric analysis [3,4] and enzymatic analysis using HPLC [9]. However, in all these cases the fraction collected contained the main component.

Here we describe the FSCE separation of some minor side products from a complex fermentation broth and the collection and identification of their enzymatic activity by reaction with two different substrates. The detection of the reaction products from the enzymatic reaction was also achieved by FSCE analysis.

EXPERIMENTAL

Materials

The fermentation sample was from a four-day-old fermentation of *A. oryzae*, made in a 10-l laboratory fermenter, with a medium containing dextrin and soy flour. The main product of the fermentation was a neutral α-amylase with pI = 4.7; the concentration of α-amylase was 1.4 g/l. The broth is known to contain at least three proteases; the total amount was approximately 200 mg as estimated by SDS-PAGE.

Prior to analysis the sample was centrifuged for 10 min at 50 *g* (Digifuge, Heraeus-Christ, Germany) to remove the mycelium, and then sterile-filtered through a 0.45-μm filter (Millipore, Molsheim, France). The sample was stored at −18°C, before analysis, to avoid degradation. The purified alkaline protease used for "spiking" was a gift from Shamkant Anant Patkar, Novo Nordisk (Bagsvaerd, Denmark).

Boric acid, sodium dihydrogenphosphate, sodium hydroxide and maleic acid for the buffers were all of analytical grade from Merck (Darmstadt, Germany). Tris-(hydroxymethyl)aminomethane (Tris) of very high purity was from Sigma (St. Louis, MO, USA).

The casein substrate contained 3.5 g/l casein "nach Hammerstein" from Merck and 0.03 g/l benzoic acid (Merck) in 30 mM Tris–maleic acid buffer (pH 7).

The Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide substrate contained 0.6 g/l Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide from Sigma in 50 mM phosphate buffer (pH 7). It should be noted that the substrate is not a real peptide, but a peptide of Ala–Ala–Pro–Phe, which is blocked at the N-terminal by succinic acid and modified by *p*-nitroanilide at the C-terminal. The *p*-nitroanilide yields a yellow colour, when not connected to the peptide. Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide is an artificial peptide substrate constructed for detection of alkaline proteases of the subtilisin family;

during protease detection the coloured free *p*-nitroanilide from the C-terminus of the peptide is released.

The FSCE analysis was performed on a Beckman P/ACE System 200 (Beckman Instruments, Palo Alto, CA, USA). The capillary was uncoated fused silica, 75 μm I.D., total length 57 cm, 50 cm to the detector, supplied by Beckman Instruments in a cartridge. The instrument was controlled and data were collected by P/ACE System 2000 software version 1.5 (Beckman Instruments) running on a Model 80 386 IBM Personal System/2 computer (IBM United Kingdom, Portsmouth, UK).

Methods

UV detection was at a fixed wavelength of 200 nm. The temperature of the capillary was maintained at 30°C by the liquid cooling system. Injection was made by applying an overpressure of 3.4 kPa to the injection end of the capillary.

The capillary was equilibrated before every run. It was first flushed for 2 min with 0.1 *M* sodium hydroxide, then for 0.3 min with water to remove the sodium hydroxide. It takes approximately 0.2 min to flush the whole capillary on this instrument (pressure 138 kPa). The water and the sodium hydroxide were flushed into a empty vial, to prevent changing the volume and pH of the buffer vial. The capillary was then flushed for 4 min with buffer, 2 min forward and 2 min reverse, to avoid changing the volume of the buffer vials when using the same buffer vials for more than one run.

For fraction collection a running buffer containing 33 m*M* phosphate at pH 9.5 was used, and 30 nl of the fermentation broth were injected. The separation was carried out using a constant voltage of 15 kV.

Before fraction collection, the sample was run normally to detect the migration time for the peak, which should be collected. The time (T_c) when the start of the peak is going to leave the capillary could then be calculated as $T_c = (57\text{ cm}/50\text{ cm}) \times T_d$, where T_d is the time when the peak passes the detector. When fractions were collected, the current was interrupted just before T_c. A new vial containing 10 μl of running buffer was placed in position at the detector end. The current was connected again and the peak forced to migrate into the collection vial. To avoid joule heating the small buffer volume in the vial, the voltage was fixed at 7.5 kV. In this case 0.6 min at 7.5 kV was used to move the peak to the collection vial.

To the vial with the collected peak, 10 μl of enzyme–substrate were added. The vial was incubated at room temperature (approximately 25°C) for 2–24 h. The conversion of substrate to product was followed simultaneously with FSCE. The casein and the Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide substrates were treated in the same way. A 100 m*M* borate buffer (pH 8.35) was used as running buffer in the FSCE analysis. An 18-nl sample was injected, and 20 kV were applied constantly during separation.

RESULTS

Separation and identification of proteases

The fermentation broth was most efficiently separated after dilution three times with deionized water using a 25 m*M* phosphate buffer (pH 9.5) as running buffer at 15 kV. Fig. 1 shows the separation with 6 nl of diluted sample injected. The main compo-

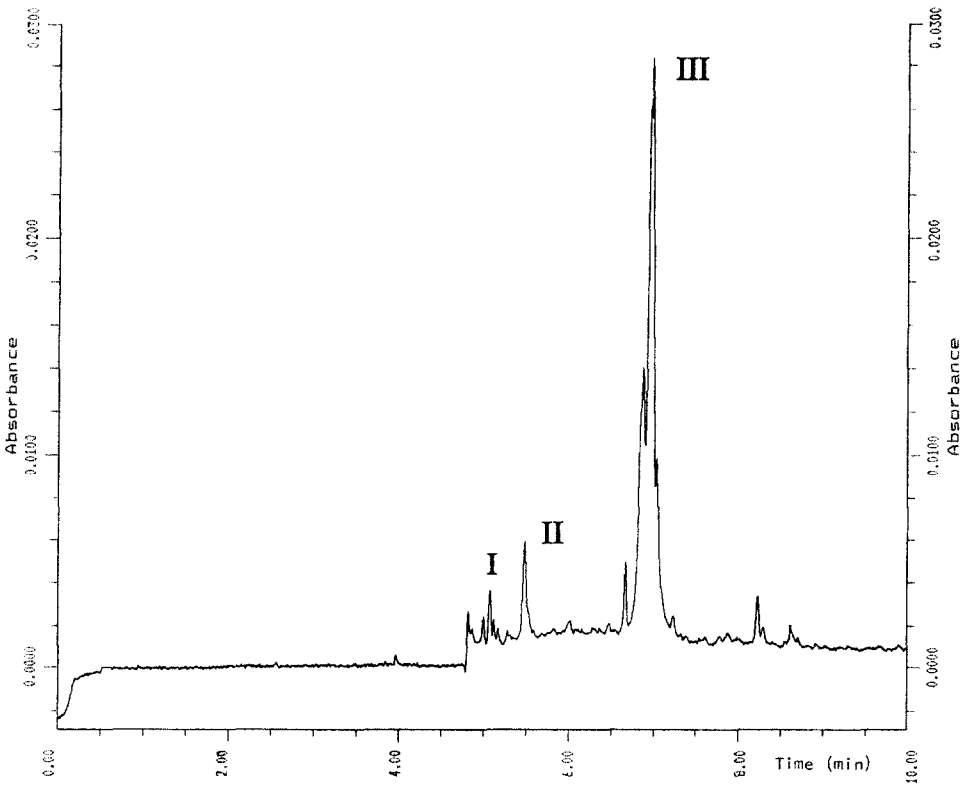


Fig. 1. FSCE pattern of fermatation broth from *Aspergillus oryzae* diluted three times with deionized water. The running buffer was 25 m*M* phosphate (pH 9.5), and a constant field of 15 kV (263 V/cm) was applied during separation. A 6-nl aliquot was injected, and detection was at 200 nm. I is an alkaline protease identified by "spiking" with a pure component, II is the peak investigated here for protease activity and III is the main product of the fermentation, a neutral α-amylase consisting of three or more non-baseline-separated peaks, caused by isoenzymes.

nent, the α-amylase (III, migration time 7 min), consists of three non-baseline-separated peaks, caused by isoezymes. The peak with migration time 5.0 min is identified as an alkeline protease (I) by "spiking" with purified alkaline protease from Shamkant Anant Patkar. The peak with migration time 5.5 min (II) is a subtilisin-like protease, identified by collection and enzymatic analysis.

Collection of fractions for identification

In order to obtain as much material as possible, undiluted broth was injected directly into the apparatus and the sample volume increased to 30 nl and the strength of the running buffer to 33 m*M*. By doing this the resolution becomes less efficient, because of greater dispersion. This is caused by the longer sample zone, the increased conductivity of the sample zone and more joule heating. To increase the amount of material further, the internal diameter of the separation capillary could also be in-

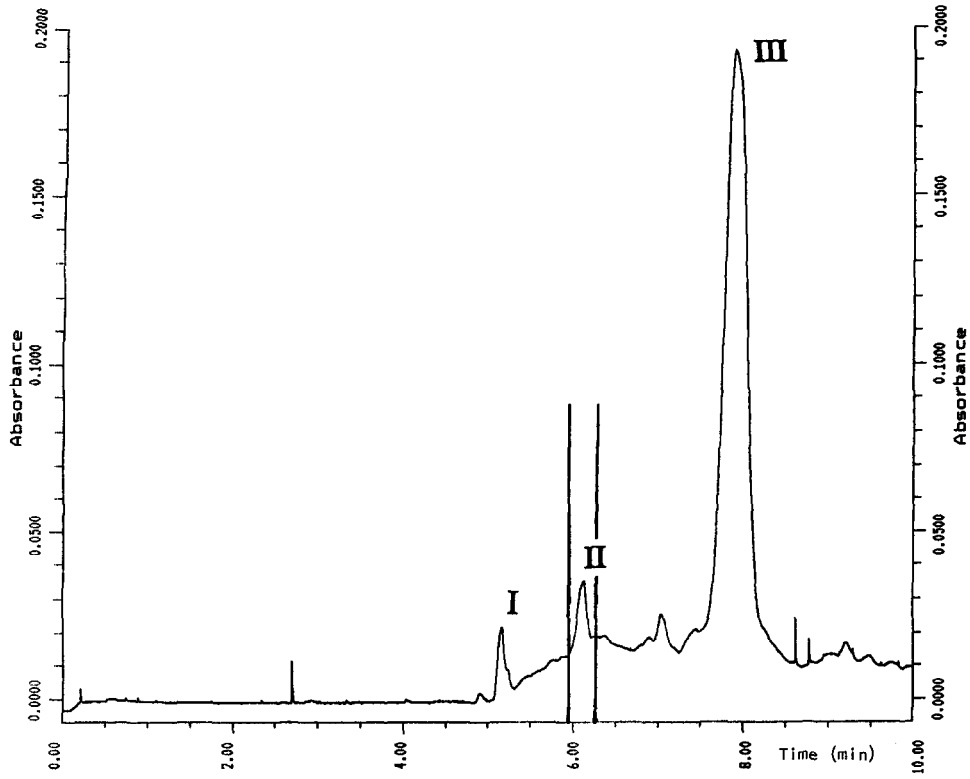
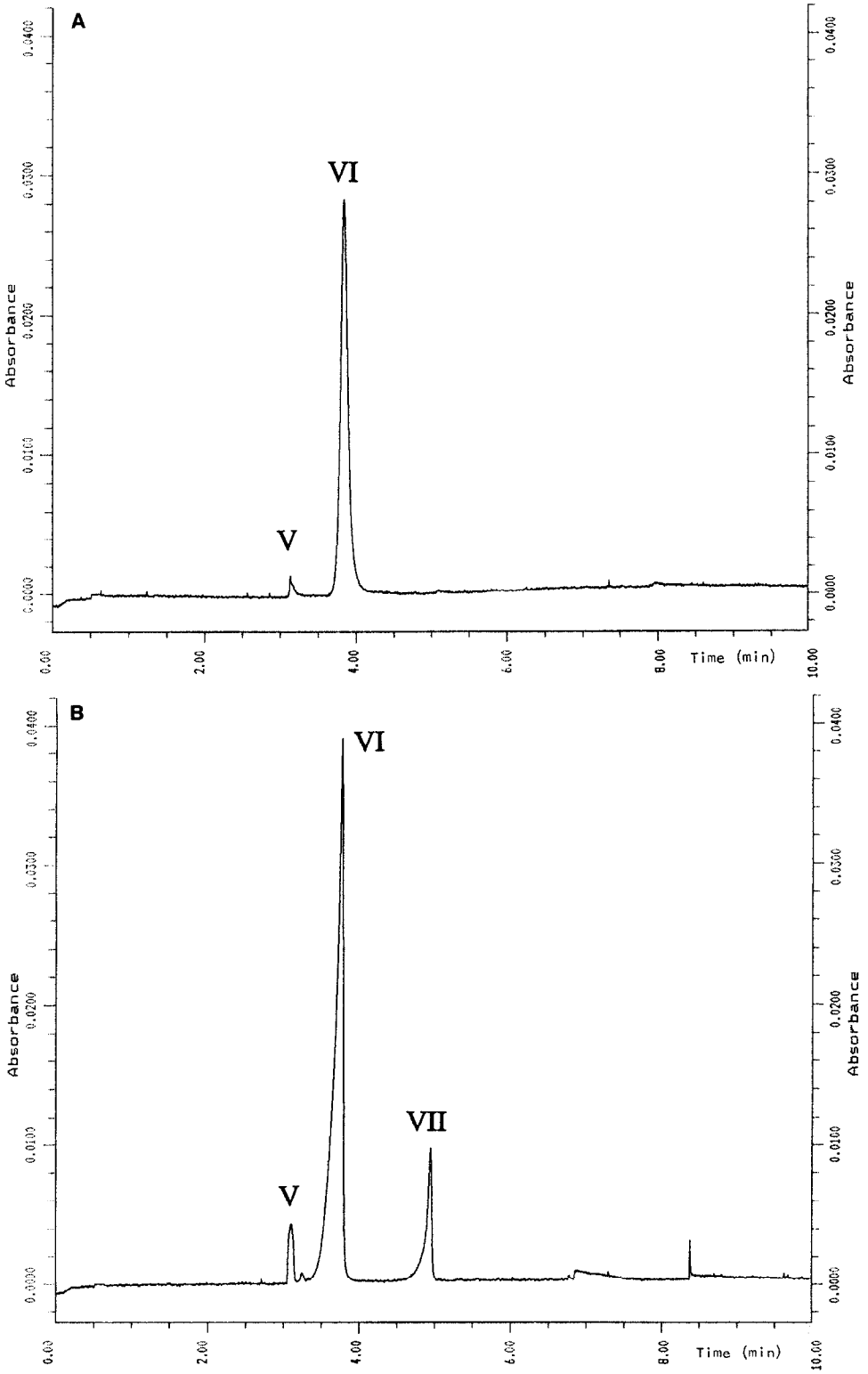


Fig. 2. FSCE pattern of fermentation broth from *Aspergillus oryzae* under fraction collection conditions. The running buffer was 33 m*M* phosphate (pH 9.5), and a constant field of 15 kV (263 V/cm) was applied during separation. A 30-nl aliquot was injected, and detection was at 200 nm. I is an alkaline protease, II is the peak investigated here for protease activity and III is the main product of the fermentation, a neutral α-amylase. The time window for collection of the peak with proteolytic activity is shown.

creased [7] or the same fraction could be collected from a number of runs [3–5], and in this way the concentration of the component in the collection vial can be increased. This was not necessary in this case, because enzymatic amplification was applied.

Fig. 2 shows the analysis of the sample using the conditions for fraction collection. The main product of the fermentation is the α-amylase (III), with a migration time of 8 min. The peak with migration time 5 min (I) is an alkaline protease identified as previously described. The peak with migration time 5.5 min (II) was also shown to possess proteolytic activity with Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide. The time window used for the collection of the peak with protease activity is aslo shown in the figure.

The reproducibility of the method was tested by collection and analysis of the same peak (peak II, Fig. 2) in four independent runs. In all cases (not shown) the protease activity detected was equal in quantity and pattern of action. The alkaline protease, identified by "spiking" (peak I, Fig. 1), was also collected, and the protease activity detected (data not shown).


A
VI
V
Absorbance
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VII
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Time (min)

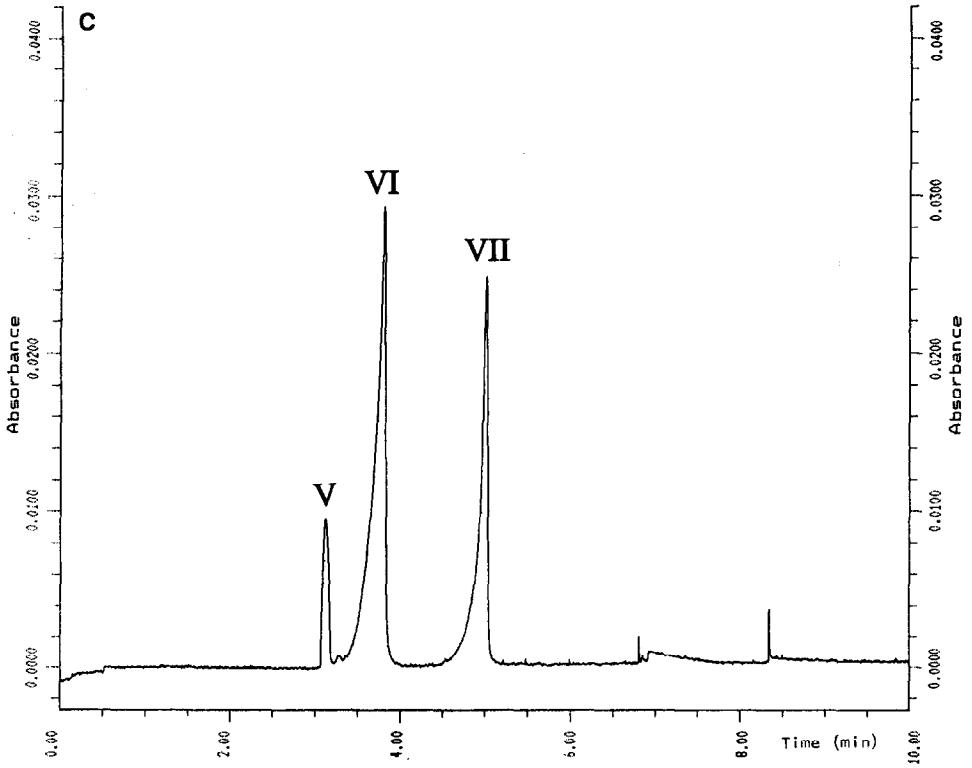

Fig. 3. FSCE enzyme assay patterns for fraction II collected by FSCE (Fig. 2), using Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide substrate. Conditions as in Fig. 2. Peaks V = *p*-nitroanilide, VI = Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide (substrate); VII = Suc–Ala–Ala–Pro–Phe. (A) Blank incubated for 8 h. (B) Incubation for 3 h. (C) Incubation for 8 h.

Enzymatic analysis of peak II (Fig. 2)

Suc–Ala–Ala–Pro–Phe–p-nitroanilide determinations. Fig. 3A–C shows the detection of proteolytic conversion of Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide (VI) to *p*-nitroanilide (V) and Suc–Ala–Ala–Pro–Phe (VII). Fig. 3A is a blank (incubated for 8 h), Fig. 3B shows the conversion after 3 h of incubation and Fig. 3C the same after 8 h. It is obvious that Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide is converted to only these two components. It is also apparent that quantitative determination is straightforward. The pattern of hydrolysis strongly indicates that peak II contains an alkaline protease of the subtilisin family, but it cannot be ruled out that it resulted from some kind of carboxypeptidase activity. To prove that the peak was due to a subtilisin-type enzyme, the proteolytic action in 100 m*M* borate buffer (pH 8.35) containing 0.6 g/l Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide and 110 mg/l Savinase (subtilisin from Novo Nordisk) 30°C, was followed with FSCE over more than 7 h as shown in Fig. 4. The irregularity of the graph at 1.6 h of incubation is an instrumental error, caused by the injection of too much sample. The disappearance of the substrate and the emergence

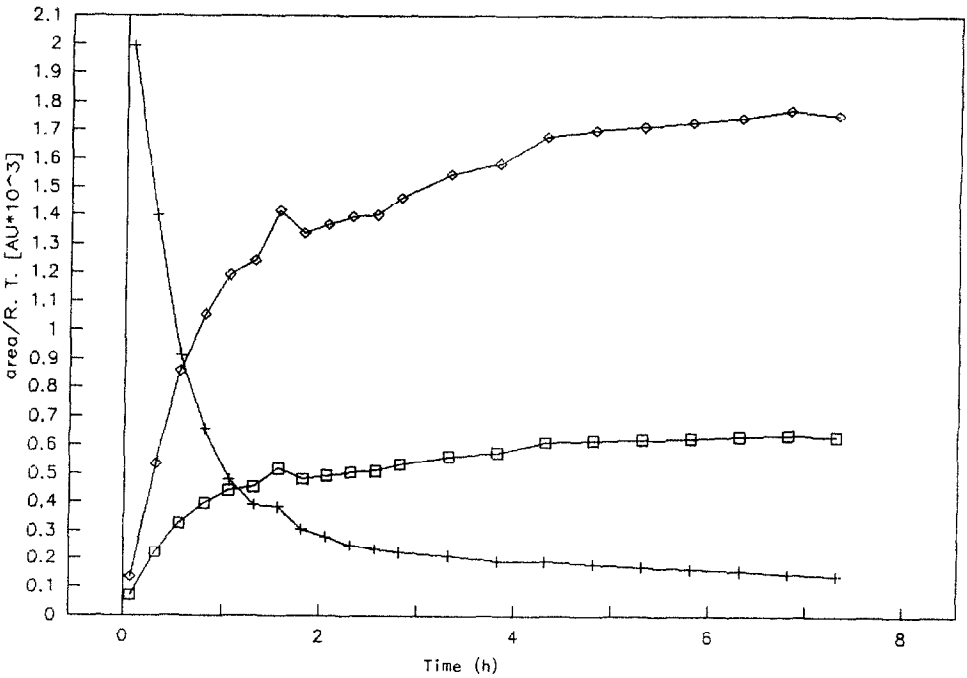

Fig. 4. FSCE enzyme assay as a function of time. Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide in 100 m*M* borate buffer (pH 8.35) containing 110 mg/l Savinase. The degradation was followed on FSCE. Conditions as in Fig. 2. + = Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide; ◇ = Suc–Ala–Ala–Pro–Phe; □ = *p*-nitroanilide.

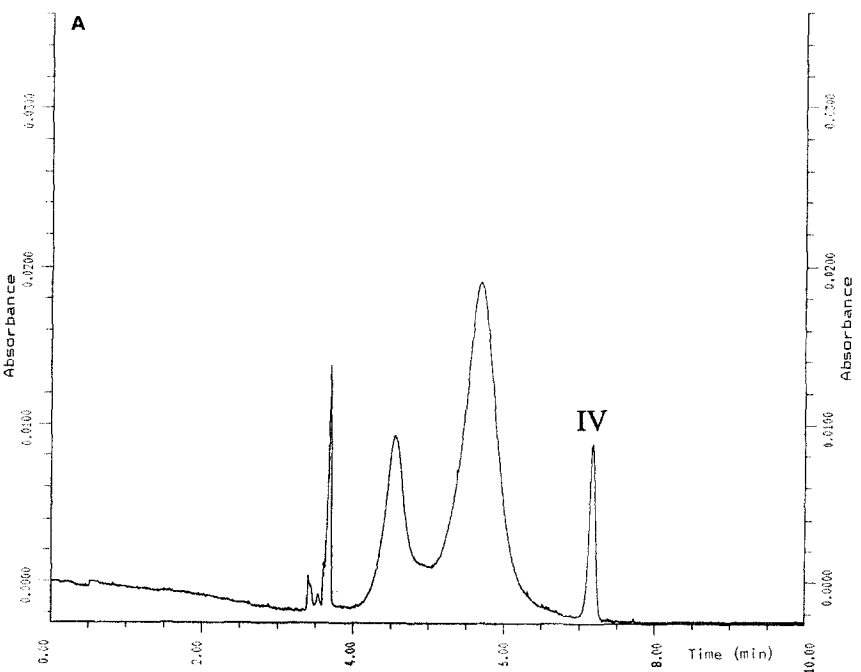

Fig. 5.

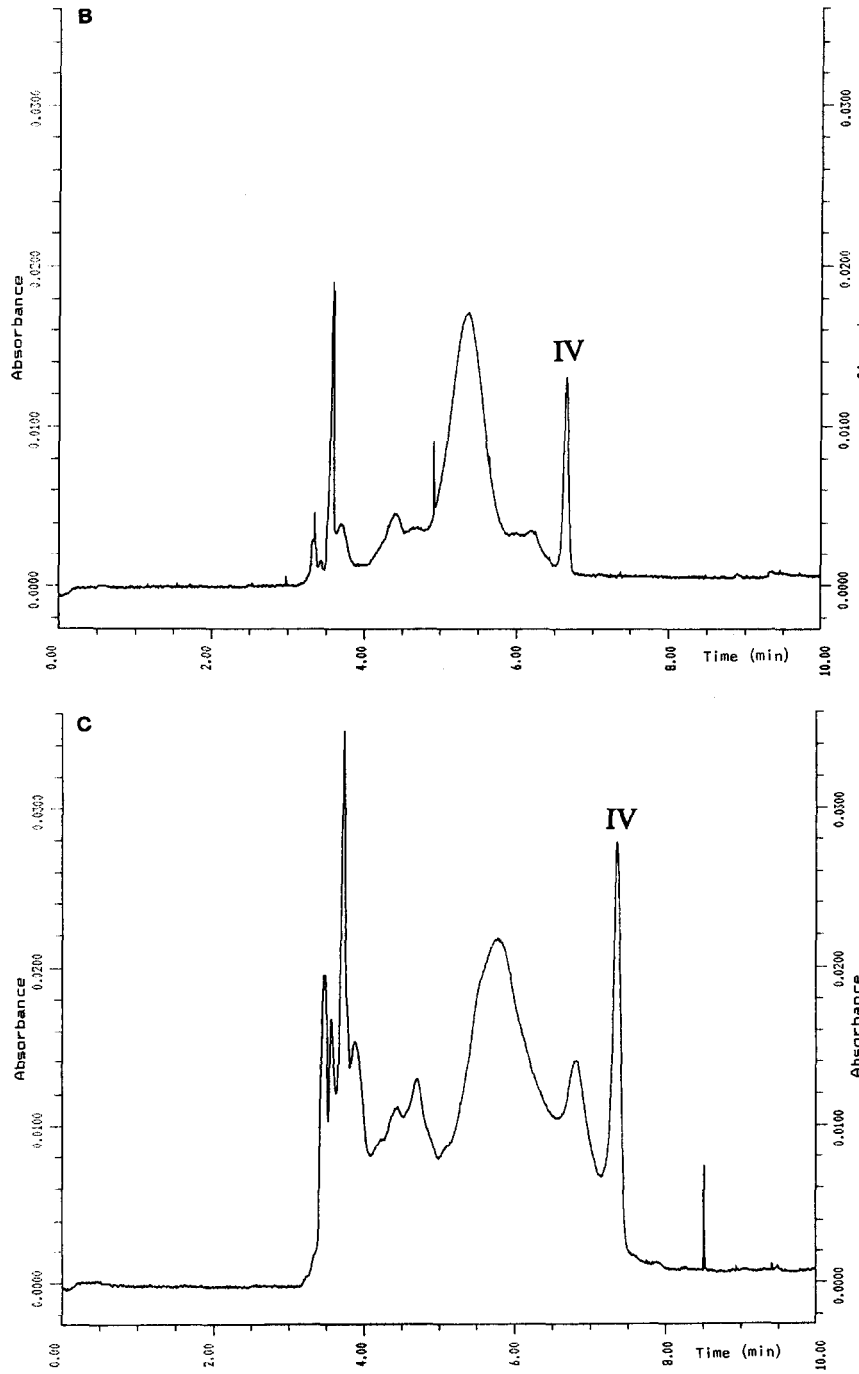

Fig. 5. FSCE enzyme assay patterns of fraction II collected by FSCE (Fig. 2), using casein as a substrate. The running buffer was 100 m*M* borate (pH 8.35), and a constant field of 350 V/cm was applied during separation. An 18-nl aliquot was injected, and detection was at 200 nm. The peak marked IV is benzoic acid, used as internal standard. (A) Blank incubated for 16 h; (B) incubation for 4.5 h; (C) incubation for 16 h.

of the products are, as expected, reciprocal events, and no internal standard is needed. There was no difference in reaction pattern between Savinase and peak II.

Casein determinations

To distinguish further between the action of a carboxypeptidase and an endoprotease, to which group the subtilisin family belongs, reactions with casein as substrate were carried out as described. The hydrolysis of casein is shown after 4.5 h (Fig. 5B) 16 h (Fig. 5C), and a blank (incubated for 16 h) is included for comparison (Fig. 5A). Benzoic acid in a concentration of 0.03 g/l was included (peak IV, Fig. 5A–C) to compensate for evaporation during the incubation. Unhydrolyzed casein is separated nicley (Fig. 5A) into two main components, the smallest of them being nearly completely degraded after 4.5 h (Fig. 5B) and both being heavily changed after 16 h of incubation (Fig. 5C), strongly confirming that peak II reacts like an endoprotease.

DISCUSSION

Throughout this work in which enzymatic activity was determined in collected fractions, blanks were included. The blanks, consisting of pure running buffer, were treated in exactly the same way as all the fractions, to take into account all changes except the ones caused by components in the fermentation broth. In particular, the changes caused by thermic degradation of the substrates could lead to wrong conclusions about protease activity if no blank was included.

Evaporation from the collection vials during the incubation was quite high, even if all vials were covered with special caps supplied by Beckman Instruments, and water was added to the bottoms of the vials to ensure high humidity around the sample. Evaporation, which increased the concentration of the substrate, increased during incubation. Benzoic acid (IV) was added as an internal standard to detect the evaporation from the sample during incubation. The peak areas increased during the incubation and varied from sample to sample, indicating that the internal standard is necessary for comparison, and that considerable evaporation occured.

Using borate as running buffer in the FSCE analysis created some difficulties with the two substrates, because the migration times varied. The capillary needed a very long time to achieve equilibrium with the borate ion, probably because borate complexes with the silanol groups of the capillary wall. The migration time was not stable even after a run of more than 20 h. Because of this only normalized areas (area/migration time) were compared [10].

The concentration of the collected protease is not known exactly, but the concentration is approximately 100 mg/l, estimated as the peak area at 200 nm (Figs. 1 and 2) and compared with the peak area of the α-amylase peak. This means that we were able to detect less than 3 ng of the protease and the concentration of the protease could under these assumptions be calculated to be 75 μg/l after substrate was added. We could probably have detected as little as 0.3 ng of the enzyme using this method.

The protease activity was detected by two different substrates. The Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide substrate was chosen because it is specific for the alkaline subtilisin-like proteases, and also because it is normally easy to quantify by colorimetric measurements. The casein substrate containing nearly all possible pep-

tide bonds is fairly unspecific and can be used to detect all kinds of proteases but can only give a semiquantitative detection of the activity in the way we used it (FSCE).

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