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Capillary electrophoretic determination of the protease Savinase in cultivation broth

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ABSTRACT

The highly basic washing enzyme Savinase and various analogues were analysed by micellar electrokinetic chromatography (MEKC) and electrophoresis. Broth samples were withdrawn during the cultivation of Savinase by recombinant microorganisms. Savinase peak areas obtained by MEKC–electrophoretic analysis were normalized with respect to migration time and compared with traditional enzyme activity measurements. The electropherograms indicated thermal degradation of the Savinase molecule at high field strengths. Baseline separation of Savinase and two analogues was achieved.

INTRODUCTION

Analytical developments are essential in order to achieve a more detailed knowledge of each of the phases present during the production of a protein by recombinant microorganisms. This includes all the steps from the cultivation process via downstream processing to the final product. During the cultivation period, analysis should be performed at frequencies that make it possible to follow the time-varying concentrations of the protein product and key metabolites. For control purposes on-line measurements are preferred.

Whereas, *e.g.*, pH, temperature and pO_2 are measured on-line on a routine basis, the concentrations of the protein product and key metabolites are most often measured off-line with a time lag which makes concentration-based control impossible. In some instances the analytical results are obtained even days after the cultivation process has been finished.

During the last 2–4 years, capillary electrophoresis (CE) has evolved into a highly efficient separation technique for the analysis of, *e.g.*, peptides and proteins [1–5]. With its simple automated instrumentation and analysis times of the order of 5–15 min, CE is an obvious choice as a potential on-line technique for the analysis of species where changes are not too significant in that time range. Furthermore, sample preparation prior to analysis can normally be reduced to centrifugation and subsequently dilution of the cultivation broth. The dilution step is used to avoid reversed sample stacking conditions (which lead to excessive peak broadening [6,7]) when high ionic strength samples (often the case with cultivation broths) are analysed.

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Generally, in CE the elution order of the analytes is manipulated by changing the running buffer composition rather than changing a column. As an example, one might determine the concentration of the protein product in one running buffer, flush the capillary with another buffer, introduce a new sample plug from the sample vial and then determine the concentration of various carboxylates and inorganic anions [8–10].

So far only a few groups have reported on the use of CE during the cultivation and downstream processing of recombinantly produced protein. Paulus and Gassmann [11] used CE to control the purity of recombinantly produced hirudin at different stages during the purification process. The full-length hirudin molecule (hirudin-65) was separated from two major degradation products, hirudin-64 and -63 (missing one and two amino acids at the C-terminus, respectively). Hurni and Miller [12] applied CE analysis to samples from each of the individual purification steps during the production of a recombinant hepatitis B vaccine expressed in *Saccharomyces cerevisiae*. Banke *et al.* [13] used a commercial CE instrument for the fraction collection of analyte bands in electropherograms of *Aspergillus* cultivation broth samples. The fractions were analysed for alkaline protease activity.

When cultivation broths are subjected to CE analysis, the electropherograms are "fingerprints" of the cultivation process at the specific time of sampling. Hence, in addition to being used to determine the actual concentrations of various identified species, the electropherograms might be used in neural nets for control purposes. In that way a knowledge-based correlation between the fingerprints and successful cultivations might be developed.

Savinase is a protease that is used as an ingredient of washing powder. It consists of 269 amino acid residues and the relative molecular mass is approximately 27 000. The isoelectric point is above 10.5 [14]. Here we report the CE analysis of cultivation broth during the production of Savinase by a recombinant *Bacillus* strain.

EXPERIMENTAL

Materials

Samples of Savinase cultivation broth and standards of Savinase and various analogues were obtained from Novo Nordisk A/S (Gentofte, Denmark). Sodium dodecyl sulphate (SDS), disodium hydrogen- and sodium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany), hydroxypropylmethylcellulose (HPMC) from Sigma (St. Louis, MO, USA) and 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) from Fluka (Buchs, Switzerland). The fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). The pH of all buffers was adjusted with either 1 *M* HCl or 1 *M* NaOH.

Methods

All the CE experiments were performed on an Applied Biosystems ABI Model 270A CE instrument. The fused-silica capillaries were total length 45 cm × 50 μm I.D. × 192 μm O.D. The distance from the introduction end to the point of detection (effective length) was 25 cm. Sample was introduced by means of a 16.8-kPa vacuum at the detector end of the capillary. An air-bath thermostated the capillary at 30°C. The electropherograms were obtained by UV absorbance detection at 200 nm. Hence, the ordinate of the electropherograms shows the absorbance and the abscissa the analysis time in minutes. Sample preparation of the Savinase cultivation broth samples prior to CE analysis consisted of centrifugation and dilution with distilled water.

The traditional determination of Savinase in culture broth is based on the hydrolysis of dimethylcasein (DMC). The primary amino groups in the peptides formed in this process react with trinitrobenzenesulphonic acid (TNBS) to form a coloured complex, which is continuously detected in order to calculate the change in absorbance per unit time [15].

RESULTS AND DISCUSSION

At neutral pH the surface of an uncoated fused-silica capillary is negatively charged. Hence, owing to its high isoelectric point, positively charged Savinase molecules adsorb on the capillary surface when CE analysis is performed in the free solution mode (FSCE) at neutral pH. One way to reduce the coulombic attraction between the Savinase molecules and the capillary surface is to titrate most of the negative surface charge off the capillary wall by lowering the buffer pH [5]. However, even in a 25

m*M* phosphate buffer of pH 1.6, tailing of the Savinase peak indicated adsorption on the capillary wall.

Another approach is to perform the analysis at a pH above the Savinase isoelectric point where it is net negatively charged. FSCE analysis was performed in a 100 m*M* CAPS buffer (pH 11.0). Concerning the sample solution, dilution of the cultivation broth with the running buffer was superior to dilution with distilled water or dilute acid with respect to resolution between the Savinase and neutral species peaks. A highly alkaline pH of the sample zone results in net negatively charged Savinase molecules migrating electrophoretically in the opposite direction of the detector and away from the neutral zone. In general, however, separation of the Savinase and neutral peaks was not satisfactory.

Addition of the anionic detergent SDS to the buffer at a sub-micellar concentration did not improve the separation.

The next approach was to perform the CE analysis in the micellar electrokinetic chromatographic (MEKC) mode [16,17]. In MEKC a detergent is added to the buffer at a concentration above its critical micellar concentration. Separation of the analytes is based on a combination of differences in electrophoretic mobilities (if the analytes are charged) and different degrees of partitioning with the charged detergent micelles. The detergent of choice was the anionic SDS. Addition of SDS improved the separation of the Savinase and neutral peaks. When a high concentration of anionic detergent molecules is added to the buffer at a pH below the protein isoelectric point, the negatively charged detergent molecule is attracted by the positively charged protein. Further, the hydrophobic tail of the detergent molecules can interact either with other detergent molecules or with the hydrophobic moieties of the protein. In this way the positively charged protein has acquired a net negative charge, thus no longer being attracted by the capillary wall. Hence, separations are based on MEKC in addition to electrophoresis. This means of dynamically coating the protein was performed at various pH values below the isoelectric point of Savinase. Figs. 1 and 2 are electropherograms of two Savinase cultivation broth samples obtained by MEKC–electrophoresis analysis in pH 7.2 (Fig. 1) and pH 9.5 (Fig. 2) SDS–phosphate buffers. Further resolution was not achieved by addition of the viscosity-increasing hydroxypropylmethylcellulose (HPMC) at a 0.05% concentration.

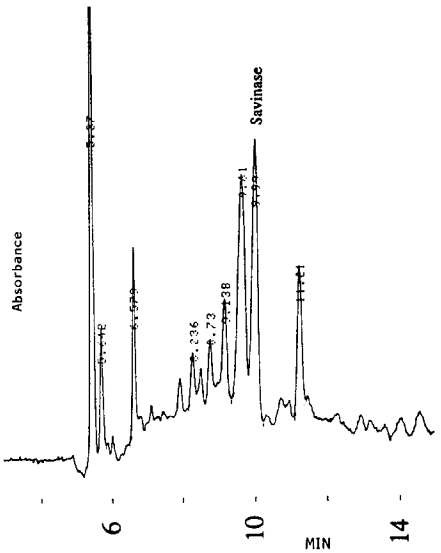

Fig. 1. MEKC–electrophoresis of Savinase cultivation broth sample I. The pH 7.2 running buffer consisted of 25 m*M* phosphate and 50 m*M* SDS. 9 kV was applied during analysis.

Covalently coated capillaries have recently become commercially available. In these capillaries the electroosmotic flow is eliminated, thus greatly reducing coulombic interactions between the ana-

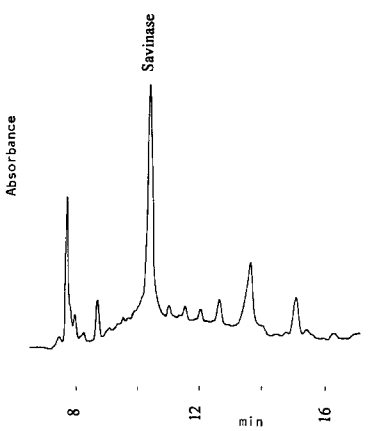

Fig. 2. MEKC–electrophoresis analysis of Savinase cultivation broth sample II. The pH 9.5 running buffer consisted of 30 m*M* phosphate and 50 m*M* SDS. 6 kV was applied during analysis.

lytes and the capillary surface. No attempts were made, however, to perform the analysis in the FSCE mode at neutral pH either in covalently or dynamically [5] coated capillaries.

Thermal degradation of Savinase at high field strengths

Fig. 3a–e show electropherograms of one Savinase cultivation broth sample obtained at 3, 9, 15,

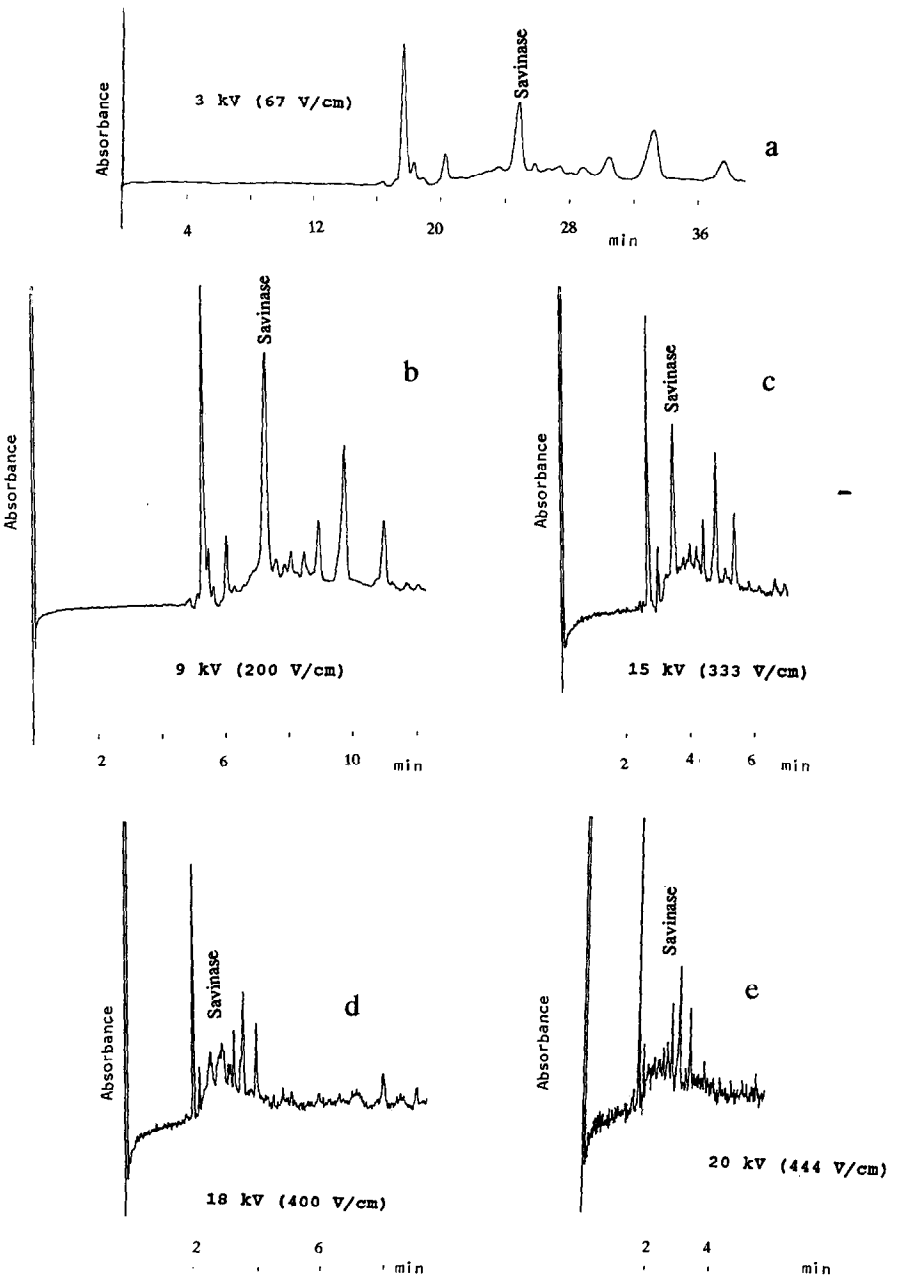

Fig. 3. Thermal degradation of Savinase at high field strengths. At high applied voltages the electropherograms indicate thermal degradation of the Savinase molecules during CE analysis. Applied potential: (a) 3; (b) 9; (c) 15; (d) 18; (e) 20 kV. The pH 9.5 running buffer consisted of 30 m*M* phosphate and 50 m*M* SDS.

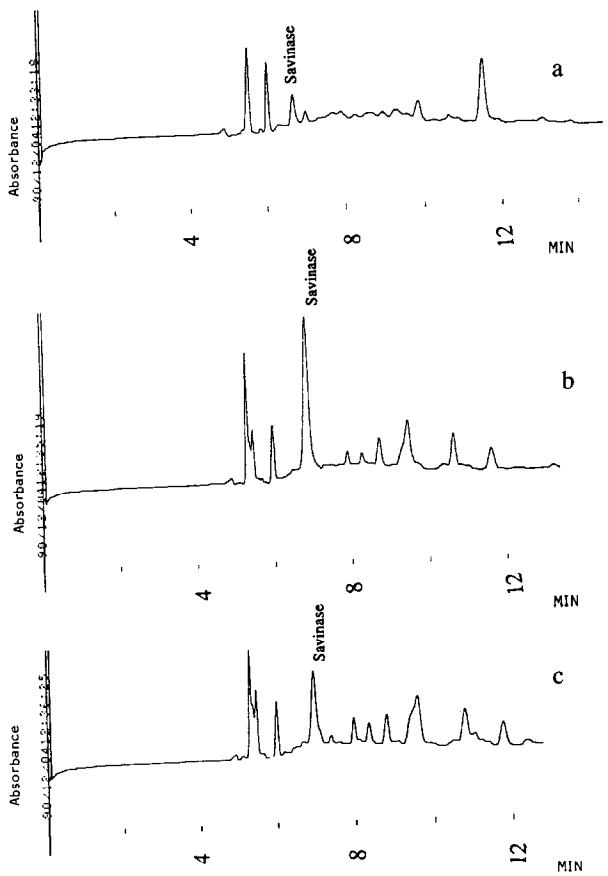

Fig. 4. MEKC–electrophoresis of Savinase cultivation broth. Relative cultivation time: (a) 7.5%; (b) 48%; (c) 100%. The pH 9.5 running buffer consisted of 30 m*M* phosphate and 50 m*M* SDS. 9 kV was applied during analysis.

18 and 20 kV, respectively, (field strength 65–450 V/cm). Stacking conditions prevailed [6,7]. The lower is the applied field strength, the larger are the peak areas owing to decreasing velocities of the analyte bands.

At potentials above 15 kV the electropherograms indicate degradation of the Savinase molecule during analysis. At 20 kV it is almost totally degraded owing to elevated temperatures in the capillary tube. At 20 kV the current was 94 μA and the power induction *ca.* 4.2 W/m. The corresponding values at 18 kV were 79 μA and 3.2 W/m. Conversion of the power values to temperature elevations of the running buffer by the use of the thermal model as described by Vinther and Søeberg [7] yielded temperatures of 50 and 45°C, respectively. As stacking conditions prevailed, the Savinase analyte zone reaches even higher temperatures while still in the originally introduced sample zone. Owing to the thermolability of Savinase, high applied voltages should therefore not be used in order to speed up analysis.

Savinase concentration vs. cultivation time

Cultivation broth was sampled six times during an extended Savinase cultivation and analysed by HPCE in the MEKC–electrophoresis mode at pH 9.5. Three of the electropherograms are shown as Fig. 4. Savinase elutes at *ca.* 7 min. In the initial phase of the cultivation period Savinase is being produced rapidly by the recombinant microorganisms. At later stages the Savinase peak area levels

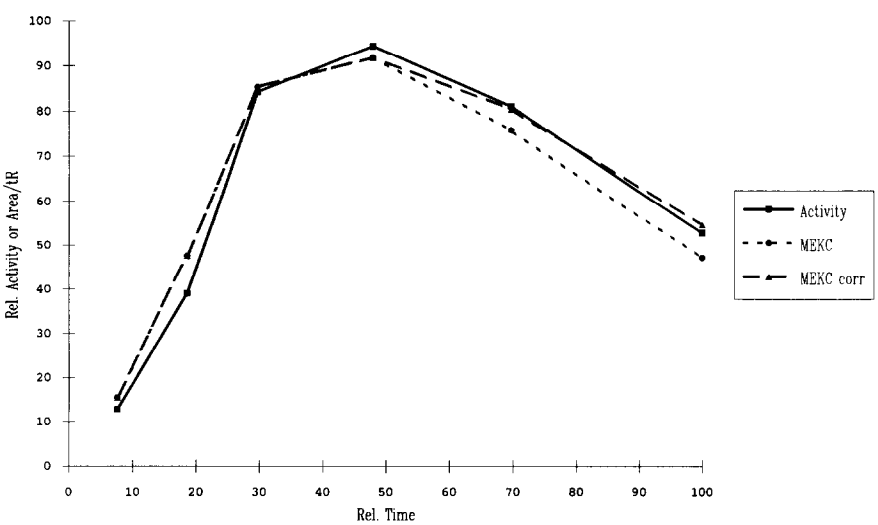

Fig. 5. Relative Savinase activity or peak area/migration time (t_R) *vs.* the relative cultivation time. See text for details.

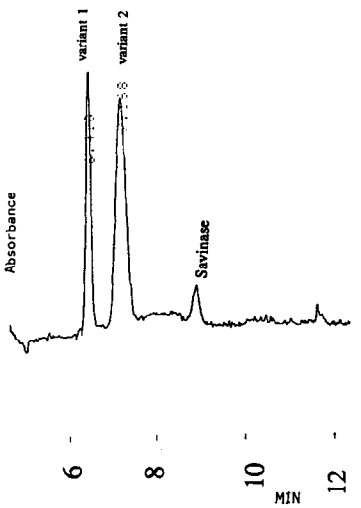

Fig. 6. Separation of Savinase and two of its variants by MEKC–electrophoresis using a 25 m*M* phosphate–50 m*M* SDS buffer pH 7.2. 8 kV was applied.

off and during the final part of the cultivation period the peak area even decreases. The reason for the decreasing peak area is probably autoproteolysis of Savinase.

The six broth samples were also analysed with respect to protease activity. Plots of relative enzyme activity and integrated Savinase peak areas (normalized with respect to migration time) are shown *versus* the relative cultivation period in Fig. 5. The Savinase peak areas were multiplied by an arbitrarily chosen constant in order to relate them to enzyme activity (at the time of analysis the Savinase standard was partly degraded). In the autoproteolysis part of the cultivation period two peaks ascended in the proximity of the Savinase peak, one on each side. If these peak areas are added to the Savinase peak area, the curve labelled MEKC corr curve in Fig. 5 is obtained; this curve agrees well with the enzyme activity curve. The integrated Savinase peak areas therefore seem to be a simple and rapidly obtained measure of Savinase activity.

Separation of Savinase variants

A sample containing Savinase and two variants of Savinase were baseline separated by MEKC–electrophoresis at pH 7.2 (Fig. 6). Variant 2 = M222A + G195E, variant 1 = G195E + H120D + K235L + *36D + N76D (amino acids numbering according to the sequence of BPN′ [18]).

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