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Short Communication

Isolation and purification of bacterial proteinases by means of autofocusing

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ABSTRACT

Autofocusing was used for the isolation and purification of neutral and alkaline proteinases from fermentation broth, after separation of cells. The yield of proteinases achieved was 19–78%, and was inversely proportional to the degree of purification, which varied from 3.0 to 7.9. Because of considerable losses of enzyme activity and the long duration of the process, autofocusing seems to be a non-economic technique for industrial isolation of relatively cheap enzymes.

INTRODUCTION

Attention has been drawn previously [1] to the possibility of industrial autofocusing as a new method for large-scale isoelectric separation of various substances and also of whole cells. Autofocusing is defined [2] as a modification of isoelectric focusing without carrier ampholytes. The pH gradient is created automatically during autofocusing by the substrate itself. The efficiency of the process depends on the conductivity of the working solution and on the concentration of the material to be separated.

We have tested autofocusing as a potential technique for isolation of bacterial proteinases.

EXPERIMENTAL

Instrumentation and conditions

An autofocuser of 1.1 dm^3 volume containing twenty cells (Technology Center of the Slovak Academy of Sciences, Košice, Czechoslovakia) was connected to a constant power supply (type VN, Development Workshops of Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

For pH and conductivity measurements a Model OK 104 pH–conductivity meter (Radelkis, Budapest, Hungary) was used.

Autofocusing was carried out at 5°C, at a power of 3 W with a variable field strength from 200 to 1000 V until the current decreased to zero. The liquid concentrate of proteinases was dissolved in pure distilled water, so that the conductivity of the solution was less than 500 μS.

Preparation of proteinases

Neutral proteinase was obtained by cultivating *Bacillus cereus* CCM 3993 in submerged culture [3]. After treatment with flocculant [4], the cells were separated by centrifugation and the supernatant containing dissolved proteinase was concentrated in a Model DC-2 ultrafilter (Amicon, Lexington, KY, USA) on an H1P10-20 Diaflo hollow fiber cartridge having a molecular weight cut-off of 5000.

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Alkaline proteinase was prepared by cultivation of *Bacillus subtilis* CCM 3701 [5] and treated in the same way as neutral proteinase.

Analytical assays

The activity of neutral proteinase (PU/ml) was determined according to Keay and Wildi [6], and the activity of alkaline proteinase (FU/ml) was assayed by the FOLP method [7] using Hammarsten casein (Serva, Heidelberg, Germany) as a substrate in both cases.

Proteins were determined by the method of Lowry *et al.* [8], using bovine serum albumin (Serva) as a standard.

RESULTS AND DISCUSSION

Autofocusing of neutral proteinase

Fig. 1 shows the separation of neutral proteinase from the filtrate of fermentation broth. Along the step-like pH gradient created during autofocusing there are two main peaks corresponding to the maximum content of proteins and to the maximum proteolytic activity. The maximum specific activity of neutral proteinase (15 000 PU/mg) was in fractions 12 and 13 with pH values from 9.8 to 10.5. The degree of purification achieved in these fractions was approximately 5.0, but the yield of enzyme was only 42% as compared with the starting activity. After analysis of all fractions we found that the loss

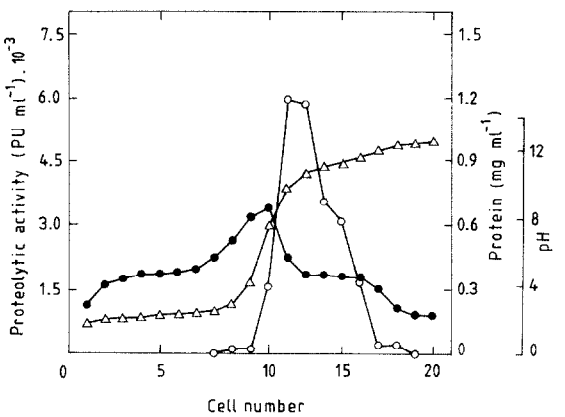

Fig. 1. Autofocusing of neutral proteinase. Processing conditions were: starting pH of the solution, 5.37; proteolytic activity, $1.7 \cdot 10^3$ PU/ml; conductivity 430 μS; time of autofocusing, 46 h. △ = pH gradient; ○ = proteolytic activity; ● = proteins.

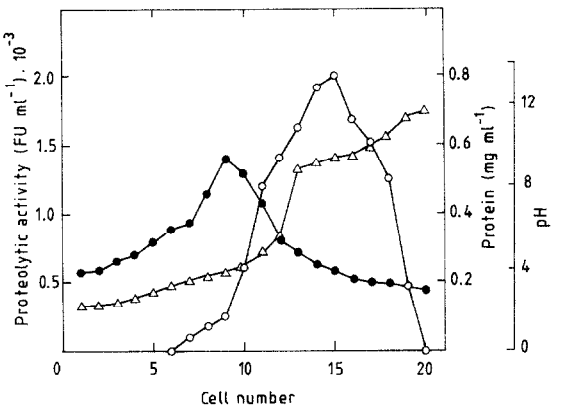

Fig. 2. Autofocusing of alkaline proteinase. Processing conditions were: starting pH of the solution, 7.44; proteolytic activity, $1.5 \cdot 10^3$ FU/ml; conductivity, 480 μS; time of autofocusing, 50 h. △ = pH gradient; ○ = proteolytic activity; ● = proteins.

of proteolytic activity due to inactivation during autofocusing was up to 19%. This phenomenon was caused by the extreme values of pH in the fractions on the border of the autofocuser, as neutral proteinase is inactivated at the pH values below 5 or above 10.

Autofocusing of alkaline proteinase

As follows from Fig. 2, the shift of the peaks corresponds to the partial separation of ballast proteins from the alkaline proteinase. However, the separation of alkaline proteinase is worse than in the case of neutral proteinase. This can be caused by large pI – pH differences in the pH gradient steps. The maximum specific activity was reached in fractions 14–16 (16 035 FU/mg) with a degree of purification of 7.9, the yield being only 19%. The pH values of this area lay in the interval from 9.2 to

TABLE I

COMPARISON OF THE AUTOFOCUSING EFFECT ON THE SEPARATION OF NEUTRAL AND ALKALINE PROTEINASES

Type of proteinase	Degree of purification	Yield (%)
Neutral	3.0	78
	5.6	42
Alkaline	5.6	40
	7.9	19

10.0 near the isoelectric point (9.4). Total loss of proteolytic activity caused by inactivation of enzyme owing to the long (50 h) effect of low pH in the fractions situated near the anode was up to 52%.

CONCLUSIONS

The results of the experiments, summarized in Table I, illustrate that autofocusing has different effects on the purification and the yield of neutral and alkaline proteinases. Partial separation of ballast proteins and the purification of both enzymes (from 3 to 7.9 times) were positive features of this process. In both cases colored impurities were focused to opposite sides of the box, so that fractions with the maximum proteolytic activity remained uncolored. Because of considerable losses of enzyme activity during this time-consuming process and the need to dialyze or to dilute the samples before separation, autofocusing seems to be an unsuitable technique for industrial recovery and purification of comparatively cheap enzymes like proteinases.

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