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Short communication

Calculation of the molecular masses of two newly synthesized thermostable enzymes isolated from thermophilic microorganisms

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Abstract

Two thermostable enzymes synthesized by thermophilic microorganisms were isolated and purified. A thermostable β-galactosidase was produced in a continuous fermentation process by *Bacillus stearothermophilus TP 32* as an intracellular enzyme. After applying different concentration procedures the raw extract enzyme was prepurified on a Sephadex G-200 size exclusion column. The isolated β-galactosidase fraction was then separated with HPLC on a TSK G 3000 SW size exclusion column to determine the molecular mass based on calibration curves of standard proteins. The other enzyme, a thermostable protease, was synthesized by *Bacillus stearothermophilus TP 26* as an extracellular enzyme. After its concentration, the enzyme was purified on a classical size exclusion column (Sephacryl S-200) and on a HPLC size exclusion column (BIO-SIL TSK-250). The micropreparatively isolated fraction was separated again on this HPLC column to determine its molecular mass. The optimum temperature of both enzymes was approximately 75°C.

1. Introduction

Extremophilic microorganisms are able to exist under extreme environmental conditions. Acidophilic Bacillus strains can live in strongly acidic (pH 1–2) as well as basic (pH 9–11) environments. On the other hand the so-called halophilic bacteria can live in saturated chloride solutions. Of special interest are the thermophilic microorganisms settling in hot sources, geysers as well as in hot water heaters.

In general proteins denature at elevated temperatures (40–55°C), but enzymes isolated from thermophilic microorganisms are more thermostable and can withstand temperatures up to 100°C without loss of their biological activity. Such thermostable enzymes are very important as catalysts for high-temperature technical processes. Selected thermophilic Bacillus strains are especially suitable in the synthesis of thermostable biocatalysts.

Their isolation can be effected extra- or intracellular followed by established "protein concentration methods", e.g. ultrafiltration, ammonium sulphate precipitation, centrifugation, polymer-phase extraction and drying of the enzyme. After classical liquid chromatographic protein purification methods high-performance liquid

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chromatography (HPLC) may be applied. Very efficient separations using size exclusion chromatography [1–9], hydrophobic interaction chromatography [10–14], ion-exchange chromatography [15–18] or affinity chromatography [19–21] have been achieved in recent years.

Using high-performance size exclusion chromatography (HPSEC), high resolutions of protein mixtures are attained in short run-times. However, one disadvantage of these columns is their low capacity. Hence, a large number of authors have described and characterized different SEC materials.

The chromatographic properties of the TSK-Gel PW type were first discussed by Hashimoto et al. [1]. Wada et al. [4] investigated protein separations on pressure-stable poly(vinyl alcohol) material (Asahipak GS-columns). Hagel and Andersson [3] evaluated the properties of a crosslinked agarose separation material and showed linear calibration graphs over a large molecular mass range (1000–4 000 000). Further studies [9] have been performed for the optimization of crosslinked procedures. The resulting Superose 6 HR has a stability of 1.5 MPa and shows linearity over the molecular range 5000–5 000 000, whereas Superose 12 HR has a comparable separation range and a pressure stability of about 4 Mpa. The elution behaviour of proteins on a crosslinked copolymer consisting of allyl-dextran and N,N-methylenediarylamide (Sephacryl S-200 HR) was investigated by Johansson and Gustavsson [8]. Anspach et al. [6] compared different SEC columns (Zorbax GF and GF 450; TSK-Gel 3000 SW and SWXL) and investigated the influence of the sodium chloride concentration of the mobile phase on the retention behaviour of standard proteins.

In our work we used the BIO-SIL TSK-250 and TSK G 3000 SW size exclusion columns for the purification and molecular mass determination of the two newly synthesised thermostable enzymes protease and β-galactosidase. These enzymes were isolated from thermophilic microorganisms and prepurified with different concentration methods, as well as with classical LC techniques.

2. Experimental

2.1. HPLC apparatus

A Shimadzu LC-6A liquid chromatograph as described previously was used [22].

2.2. Stationary and mobile phases

The HPSEC columns used were a 600×7.5 mm I.D. BIO-SIL TSK-250 and TSK G 3000 SW (Bio-Rad Labs, Richmond, CA, USA).

Double distilled water, Na_2SO_4, Na_2HPO_4, NaCl, HCl, $(NH_4)_2SO_4$ (Laborchemie Apolda, Germany) were used for the preparation of the buffers.

2.3. Standard proteins and calibration curve

Thyroglobuline, immunoglobulin, ovalbumin, myoglobin, cyanocobalamine, and DNP-alanine were used to prepare calibration curves. Thyroglobuline is excluded and presents the so-called intermediate particle volume, whereas the amino acid DNP-alanine passed through the whole particle volume of the stationary phase and is noted as the void volume [6,23].

The chromatograms showed mostly baseline separation between standard proteins of different molecular masses which results in narrow peaks. Immunoglobulin, however, is characterised by strong peak broadening. As discussed by Meng et al. [7] this may be the result of impurities in this standard protein. Whereas a linear behaviour is observed for the molecular mass range between thyroglobuline (M_r = 660 000) and myoglobin (M_r = 17 000), lower-molecular-mass compounds such as cyanocobalamine and DNP-alanine show considerable deviations from the calibration curve. Hence, molecular mass determinations of proteins smaller than 10 000 are not accurate.

Nearly identical standard protein separations are achieved with the comparable size exclusion column TSK G 3000 SW.

2.4. Isolation and prepurification of the thermostable enzymes

Two thermophilic Bacillus strains (*Bacillus stearothermophilus TP 26 and TP 32*) were selected, characterized and used for the synthesis of a thermostable protease and β-galactosidase. The protease was produced in a continuous fermentation process as an extracellular enzyme. For the isolation of the β-galactosidase, which as an intracellular enzyme is located in the cell interior, an additional lysozyme treatment was necessary. The main steps for the isolation, concentration and purification of both enzymes are listed in Table 1.

An example of the prepurification steps (ammonium sulphate precipitation, dialysis and classical column chromatography on Sephadex G-200) for the thermostable β-galactosidase with respect to protein concentration, specific activity and purification factor is presented in Table 2. These steps can be further optimised to achieve higher β-galactosidase activity.

Table 1
Isolation, concentration and purification steps for the thermostable protease and β-galactosidase

Protease	β-Galactosidase
Bacillus stearothermophilus TP26	*Bacillus stearothermophilus TP32*
Continuous fermentation process of an extracellular enzyme	Continuous fermentation process of an intratracellular enzyme
Ultracentrifugation Separation of the biomass, washing	Lysozyme treatment Ammonium sulphate precipitation
Ammonium sulphate precipitation Dialysis	Washing, drying
Acetone precipitation Lyophilization	Classical SEC: Sephadex G 200
Classical SEC: Sepharcryl S-200	HPIEC: DEAE
HPSEC: BIO-SIL TSK-250 600 × 7.5 mm I.D.	HPSEC: TSK G 3000 SW 600 × 7.5 mm I.D.

Table 2
Example of prepurification steps for β-galactosidase according to protein concentration, specific activity and purification factor

Step	Protein concentration (mg/ml)	Specific activity (U/mg)	Purification factor
Raw extract	30	6	1
Ammonium sulphate precipitation	20.7	12	2.1
Dialysis	5	29	4.8
Sephadex G 200	1.6	60	10

2.5. Determination of protease activity

The proteolytic activity of the enzyme solution was determined from degradation of the chromogenic substrate azocasein. The absorbance at 440 nm of the resulting trichloroacetic acid soluble degradation products was measured as described previously [23].

2.6. Determination of β-galactosidase activity

The hydrolytic degradation of *o*-nitrophenyl-β-D-galactoside (ONPG) to galactose and *o*-nitrophenol by β-galactosidase was used as a measure of the enzyme activity. The absorbance of the resulting *o*-nitrophenol solution was measured at 420 nm [23].

3. Results and discussion

3.1. Molecular mass determination of protease and β-galactosidase

The BIO-SIL TSK-250 and TSK G 3000 SW size exclusion columns were used for molecular mass determinations of the thermostable enzymes. Fig. 1 shows the separation of a purified

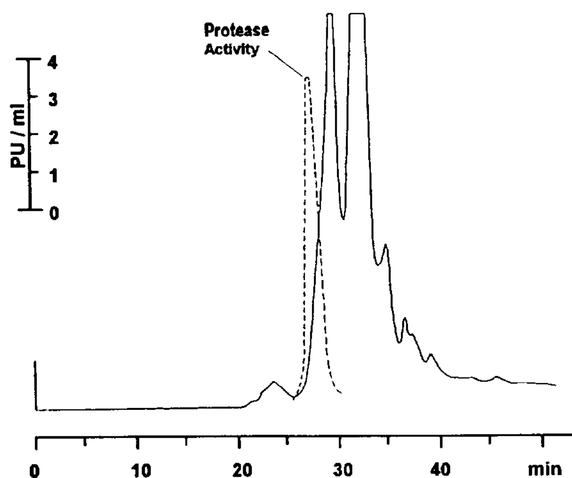

Fig. 1. Chromatogram and activity curve of a purified protease raw extract. Column: BIO-SIL TSK-250, 600 × 7.5 mm I.D.; mobile phase: 0.05 M Na_2SO_4 + 0.02 M NaH_2PO_4 (pH 6.8), flow-rate: 0.7 ml/min, pressure: 2.5 MPa; detection: UV, 280 nm; attenuation: 5; injection volume: 200 μl.

protease raw extract on a BIO-SIL TSK-250 column. Since many impurities, mainly of low-molecular-mass, are detected molecular mass determination for the protease in such a complex mixture is very difficult. The main protease activity was measured between 28 and 29 min. This protease fraction was micropreparatively isolated and separated again on the SEC column under the same chromatographic conditions. The result is shown in Fig. 2, where the main protease activity was measured after 28.4 min.

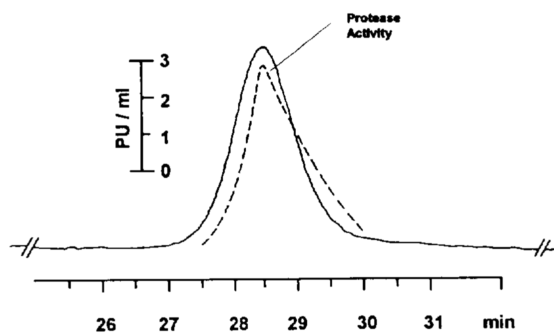

Fig. 2. Chromatogram of the "rechromatography" of a protease fraction isolated between 28 and 29 min as shown in Fig. 1.

Based on the calibration curve for standard proteins a molecular mass of 17 000 was determined for this thermostable protease.

For the molecular mass calculation of β-galactosidase a TSK G 3000 SW size exclusion column was used. The separation of a prepurified β-galactosidase extract on this SEC column is illustrated in Fig. 3.

After measuring the enzyme activity of collected fractions in small portions (volume: 200 μl) and comparing their retention volume with that of the calibration curve, a molecular mass of ca. 257 000 was calculated.

A higher precision can be achieved if a SEC column with a larger separation range, e.g. a

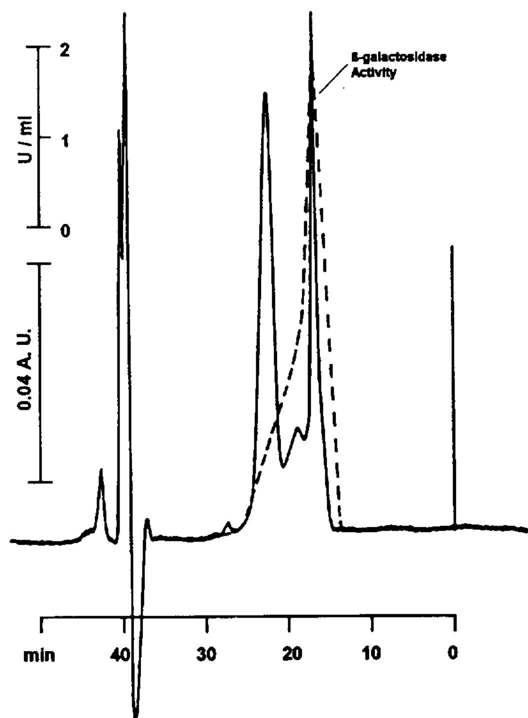

Fig. 3. Chromatogram and biological activity curve of a prepurified β-galactosidase extract. Column: TSK G 3000 SW, 600 × 7.5 mm I.D.; mobile phase: Sörensen phosphate buffer (pH 6.8) + 0.1 M NaCl, flow-rate: 0.5 ml/min, pressure: 1.8 MPa; detection: UV, 254 nm; attenuation: 5; injection volume: 200 μl.

TSK G 4000 column, is used, because this enzyme is eluted near the exclusion volume of the column.

3.2. Characteristics of the thermostable enzymes

Fig. 4 shows the dependence of the β-galactosidase activity on the sodium chloride concentration. A 1-mg amount of enzyme preparation was diluted in 1 ml NaCl solution in 1 molar steps. Up to a concentration of 3 *M* NaCl the recovery of β-galactosidase activity was nearly 100%. Higher NaCl concentrations showed a dramatic loss of β-galactosidase activity.

To determine the optimum temperature of the purified thermostable β-galactosidase, a 1-mg amount of enzyme preparation was added to 1 ml Tris-HCl buffer (pH 7.0) and the enzyme activity was measured between 40 and 100°C. As seen in Fig. 5 at 75°C the β-galactosidase activity was found to be nearly 100%, whereas at 80°C and higher temperatures a strong decrease in enzyme activity is observed. The thermostable protease showed a comparable dependence on temperature, the optimum being found between 75 and 80°C [24].

Thus the thermostability of both enzymes isolated from thermophilic microorganisms, prepurified and purified as described above and characterised according to their molecular masses by high-performance size exclusion chromatography could be clearly demonstrated.

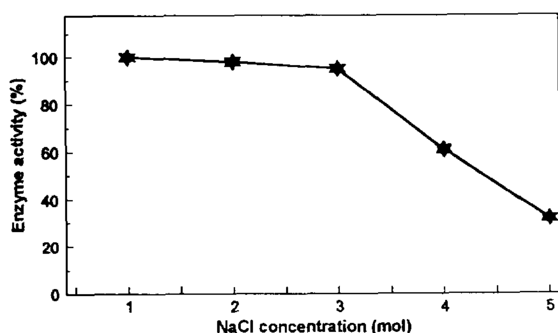

Fig. 4. Dependence of β-galactosidase activity on sodium chloride concentration (1–5 *M*). Experimental conditions: see text.

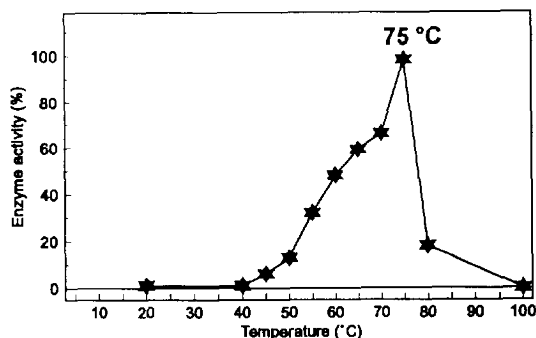

Fig. 5. Dependence of β-galactosidase activity on temperature (40–100°C). Experimental conditions: see text.

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