A rapid and simple method for the purification of transglutaminase from *Streptoverticillium mobaraense*

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Transglutaminase from *Streptoverticillium mobaraense* was partially purified by ion-exchange chromatography on a weak acid material and hydrophobic chromatography. The separation with a strong acid ion-exchanger produces homogeneous transglutaminase, in a single step and with high yields, directly from the centrifuged and filtered culture fluid of the micro-organism. The procedure reproduced several times could be also carried out on a larger scale with the optimized parameters of the laboratory isolations. The purified enzyme demonstrated good storage stability.

INTRODUCTION

Transglutaminases (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) belong to a category of enzymes which possess the ability to modify proteins post-translationally [1–3]. They catalyse the reaction in which the acyl residue of the γ-carboxamide group of peptide- or protein-bound glutamine is transferred to a primary amine. When ε-amino groups of endo-lysine residues take part in the transfer reaction, this may result in the formation of N^{ϵ}-(γ-glutamyl)lysine isopeptide cross-links. The hydrolysis of the γ-carboxamide group of glutamine catalysed by transglutaminase plays a subordinate role and takes place at low levels or in the absence of an appropriate amine [4–6]. A transasparaginase which may catalyse analogous reactions of the β-carboxamide group of protein-bound asparagine could not be detected so far, in spite of the evidence of N^{ϵ}-(β-aspartyl)lysine isopeptide cross-links in proteins [7,8].

The transglutaminases are widely distributed in organisms [1], but until recently it could only be demonstrated that they exist in animal tissue [9]. The animal enzymes need Ca^{2+} as a cofactor for the transfer reaction. The most extensively investigated transglutaminase is the human plasma protein Factor XIII, because of its important role in blood clotting, as well as guinea-pig liver transglutaminase, because of its high level in that tissue [1,6].

The inexpensive and broad use of transglutaminase certainly requires an enzyme which is sufficiently stable and comes from a reliable source. Most applications were carried out with guinea-pig liver transglutaminase. The recombinant enzyme could be obtained successfully from *Escherichia coli* [10]. However, the application of this enzyme may be limited because of its moderate stability resulting from 17 cysteine residues, none of which is involved in disulphide bridges [11]. Of greater interest for applications in protein chemistry seems to be a microbial transglutaminase recently isolated from the culture filtrate of a strain of *Streptoverticillium* sp. [12]. In contrast with the hitherto-known enzymes, this transglutaminase does not need Ca^{2+} as cofactor. It possesses a pI of 8.9 and a low molecular mass of about 40 kDa. The substrate specificity is lower than that of guinea-pig liver transglutaminase, whereas the thermal stability is greater. It is especially these last two properties which might provide for a broader application of the enzyme.

Ando et al. [12] have described a procedure for the purification of the microbial transglutaminase without giving further details. During our investigations we found a step involving ion-exchange chromatography on Amberlite CG-50 very difficult to reproduce. We are therefore presenting here a rapid and simple single-step method that allows the reproducible production of transglutaminase with high purity and in very good yields from the culture filtrate of *Streptoverticillium mobaraense*.

EXPERIMENTAL

Materials

Cbz-L-glutaminylglycin (*N*-carbobenzoxy-L-glutaminylglycin) was obtained from Bachem (Heidelberg, Germany), L-glutamic acid γ-monohydroxamate and Amberlite CG-50 from Sigma (Deisenhofen, Germany), BSA and preprepared gels for isoelectric focusing (i.e.f.), Servalyt Precotes (pH 3–10) from Serva (Heidelberg, Germany), as well as phenyl-Sepharose and SDS/PAGE gels (Excel Gel) from Pharmacia (Freiburg, Germany). Potato starch was of commercial quality. The remaining chemicals of analytical-reagent grade, nutrients for cultivations and Fractogel EMD SO_3^- (S-material with bead size of 0.02–0.04 mm and M-material with 0.045–0.09 mm) were from Merck (Darmstadt, Germany).

Organism

Streptoverticillium mobaraense was obtained from the Deutsche Sammlung für Mikroorganismen (D.S.M.) (Braunschweig, Germany). Freeze-dried cells were placed in 2 ml of sterile water following the procedure recommended by D.S.M. For the formation of spores, an inoculum was spread on Petri dishes using agar media containing malt extract. After cultivation for 6–8 days at 30 °C, the spores were collected with 5 ml glycerol/water (1:1, v/v). The resulting suspension was used for the further operations or stored at −20 °C.

Production of transglutaminase

The micro-organism was cultivated as described by Ando et al. [12]. In 2-litre Erlenmeyer flasks, 0.1 ml of the spore suspension

Abbreviations used: Cbz, *N*-carbobenzoxy; i.e.f., isoelectric focusing; D.S.M., Deutsche Sammlung für Mikroorganismen.
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was grown in 500 ml of a medium containing: polypeptone, 2.0%; yeast extract, 0.2%; K_2HPO_4, 0.2%; $MgSO_4,7H_2O$, 0.1%; potato starch, 2.0%; glucose, 0.5%; pH 7.0. Cultivation was continued at 30 °C, the culture being aerated and shaken at 90 rev./min for 9–11 days until the maximum of enzyme activity was reached. The culture fluid was separated from the mycel by centrifugation at 10000 *g* for 10 min and after filtration over a folded filter paper. The supernatant was directly utilized in the chromatography or stored at −20 °C.

For scaled-up growth, 1.0 ml of the cell suspension was transferred to 3.5 litres of a medium containing 2.5% potato starch instead of 2.0%, and 1.0% glucose instead of 0.5%. The culture was grown with aeration (2 litres/min) and stirring at 140–180 rev./min for the first day and subsequently at 300–350 rev./min. Maximum of enzyme activity was achieved after 7 days.

Measuring the transglutaminase activity

Hydroxamate formation of Cbz-L-glutaminylglycin was measured by the colorimetric procedure described by Grossowicz et al. [13], using L-glutamic acid γ-monohydroxamate for the calibration curve. Protein concentrations were determined by the method of Lowry with BSA for standardization [14]. To determinate $V_{max.}$ and K_m, a Lineweaver–Burk plot were constructed from rates obtained within the first 10 min of hydroxamate formation.

Electrophoresis

Samples of transglutaminase preparations were analysed by SDS/PAGE [15], followed by silver staining. To estimate the molecular mass of transglutaminase, a standard kit from Sigma was used. The pI was determined following Serva's method (staining with Coomassie Blue). To identify the transglutaminase band, a gel consisting of 10% agarose with 15 mM Cbz-L-glutaminylglycin and 0.1 M hydroxylamine in 0.2 M Tris buffer, pH 6.0, was spread over the i.e.f. gel for 30 min at room temperature, followed by staining with 12% HCl/5% $FeCl_3,6H_2O$ (in 0.1 M HCl)/12% trichloroacetic acid (1:1:1, by vol.).

Apparatus

The instrumentation for the purification of transglutaminase comprised the following: standard chromatography apparatus with gradient mixer GM-1, peristaltic pump P-1, single-wavelength-detector UV-1 and fraction collector FRAC-100 (from Pharmacia, Freiburg, Germany); h.p.l.c. apparatus with 'intelligent' pump L-6210, UV/VIS-detector L-4200, fraction collector L-5200, GPC-integrator D-2520; pilot plant Prep Bar Bio 100 E (all from Merck, Darmstadt, Germany).

RESULTS AND DISCUSSION

Purification of transglutaminase by ion exchange chromatography on Amberlite CG-50 and hydrophobic chromatography on phenyl-Sepharose

In our hands the method described by Ando et al. [12], for the isolation of transglutaminase from concentrated culture fluid with the weak acid ion-exchanger Amberlite CG-50 cannot be reproduced without modifications. Problems arose especially with the ion-exchange material. This could only be adjusted to a constant pH value with difficulty, and it was also subject to great changes in volume. The best results were obtained by equilibrating Amberlite CG-50 in a beaker with 50 mM sodium phosphate buffer, pH 6.5 (4 and 5 times the volume of the ion-exchange material), stirring occasionally and correcting the pH with 1 M NaOH or 1 M HCl. The buffer was changed two or three times, and a 2.6 cm × 23 cm column was packed with the equilibrated material. These, and the following steps of the procedure, were carried out at temperatures below 5 °C. In a typical experiment, 350–400 ml of centrifuged and filtered culture fluid with an activity level of 1.5–2.0 units/ml, pH 6.0, and subsequently 400–500 ml of 50 mM phosphate buffer, pH 6.5, for the elution of unbound proteins (between 80 and 90% of total proteins) were pumped into this column. The use of culture fluid concentrated by ultrafiltration was not necessary for the procedure. Chromatography took place at a flow rate of 3.5 ml/min and with a linear increasing gradient of NaCl from 0 to 0.5 M. Fractions (5 ml) were collected and examined for enzyme activity. Those with activity greater than 0.8 unit/ml were pooled. The maximum enzyme activity was found at the downward flank of a broad protein peak (Figure 1). With this method we could obtain transglutaminase in five reproduced chromatographies with an average yield of about 50% and a specific activity of 2 to 4 units/mg. The enzyme was purified 10–17-fold. The results of one such representative chromatography are summarized in Table 1.

For the further purification of transglutaminase, solid $(NH_4)_2SO_4$ was added up to a concentration of 1 M to the combined active fractions of the ion-exchange chromatography. The resulting salt-rich fluid was charged on a 1.6 cm × 29 cm column of phenyl-Sepharose previously equilibrated with 1.0 M $(NH_4)_2SO_4$ in 0.5 M sodium phosphate buffer, pH 6.5. After the washing stage, with 100–150 ml of this buffer, elution was carried out at a flow rate of 1.7 ml/min with 350 ml of a linear decreasing gradient of 1.0 M $(NH_4)_2SO_4$ in 0.5 M phosphate buffer, pH 6.5, to 0 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 6.5 (Figure 2). The eluent was collected in 5 ml portions. Fractions with enzyme activity greater than 0.5 unit/ml were combined. In five experiments we could obtain transglutaminase with low losses and specific activities of 9–14 units/mg.

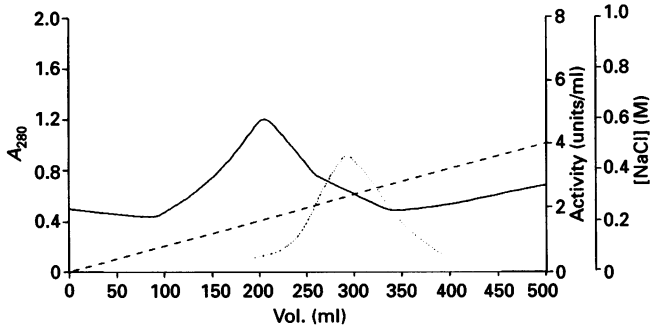

Figure 1 Chromatographic separation of culture fluid of *Streptoverticillium mobaraense* containing transglutaminase on Amberlite CG-50 at pH 6.5 and < 5 °C

Elution of protein, monitored at 280 nm (——) was achieved by a linear gradient of NaCl from 0 to 0.5 M in 50 mM sodium phosphate buffer (------). Fractions (5 ml) were collected. The enzyme activity (· · · · · ·) was measured with Cbz-L-glutaminylglycine.

Table 1 Purification of transglutaminase using ion-exchange chromatography on Amberlite CG 50 and hydrophobic chromatography on phenyl-Sepharose

Purification step	Volume (ml)	Total protein (mg)	Total units	Specific activity (units/mg)	Recovery of enzyme activity (%)
Culture fluid	230	1757	495	0.28	100
Ion-exchange chromatography	131	83	248	3.0	50
Hydrophobic chromatography	102	n.d.	214	n.d.*	43
Ultrafiltration	34	22	197	9.0	40

* n.d.: not determined.

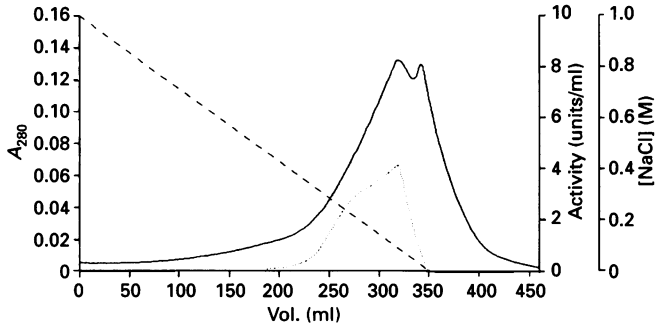

Figure 2 Hydrophobic chromatography of transglutaminase on phenyl-Sepharose with a linear gradient of $(NH_4)_2SO_4$ in 0.5 M sodium phosphate buffer, pH 6.5, and < 5 °C

Fractions (5 ml) were collected. ——, A_{280}; ------, $(NH_4)_2SO_4$ gradient; · · · · · ·, enzyme activity.

Purification of transglutaminase by ion-exchange chromatography on Fractogel EMD SO_3^-

The purification of transglutaminase by ion-exchange chromatography on Amberlite CG-50 with the accompanying hydrophobic chromatography on phenyl-Sepharose is very time-consuming, and the enzyme preparations obtained are even then not completely free of other proteins (Figure 4 below). For most applications this degree of purity may be sufficient. The ion-exchange chromatography on Amberlite CG-50 remains unreliable. The pH fluctuations, inexplicable in most cases, often gave rise to a spectacular decrease in the transglutaminase-binding capacity of the material during column charging or to bad resolution of protein elution. We found, then, more than 50 % of the enzyme in the fore-run, and enzyme activity in nearly all the fractions after increasing the ionic strength (*1*).

A very selective and strict separation of transglutaminase of *Streptoverticillium mobaraense* may be achieved with the strong ion-exchange material Fractogel EMD SO_3^-. By utilizing a low-pressure apparatus, we could work out a rapid procedure that no longer required the cooling of the columns. As in the previous procedure, centrifuged and filtered culture fluid with an activity of 1.5–2.0 units/ml, pH 6.0, was directly pumped, without prior concentration, into a 1 cm × 15 cm column which had been previously equilibrated with 50 mM sodium phosphate buffer, pH 6.0. After application of the sample, the column was washed with 40 ml of the same buffer. The most effective separation of the bound proteins was obtained at a flow rate of 1 ml/min by elution with 100 ml of a linear increasing gradient of 0–0.5 M NaCl in 50 mM phosphate buffer, pH 6.0 (Table 2). In seven chromatographies we could reach an average enzyme recovery of about 60 % and enzyme activities of 11.3 units/mg. Transglutaminase was eluted as the last protein at 0.2–0.3 M NaCl (Figure 3).

This procedure could be reproduced on a larger scale without significant modifications. The use of stepwise gradients that were necessary for the larger-scale approaches (columns with diameters of 10 and 20 cm, depending on the equipment) did not represent any disadvantage in the procedure. The obtained average values of enzyme recovery and activity using a 2.6 cm × 14 cm column (chromatographies: 11; flow rate: 6.5 ml/min; volume of gradient buffer 690 ml), a 10 cm × 14 cm column (3; 130 ml/min; 15 l) and a 20 cm × 14 cm column (1; 400 ml/min; 50 l) varied between 54 and 77 % respectively between 9.9 and 12.0 units/mg. The best result was with a yield of 59 %, a homogeneous preparation of transglutaminase with activity of 17.6 units/mg (Figure 4). The enzyme was purified approx. 128-

Table 2 Representative results of different chromatographies on Fractogel EMD SO_3^- (S-material) at the purification of transglutaminase

Column diameter (cm)	Volume (ml)	Culture fluid		Recovery of transglutaminase			
		Total units	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Purification factor	Yield (%)
1	15.0*	45.0	0.24	25.3	13.9	58	56.2
2.6	130	312	0.23	163.8	12.4	54	52.5
10	1800	3888	0.19	2806	10.4	54	72.2
20†	4100	4346	0.13	3323	9.9	74	76.5

* Concentrated culture fluid.
† Use of M-material.

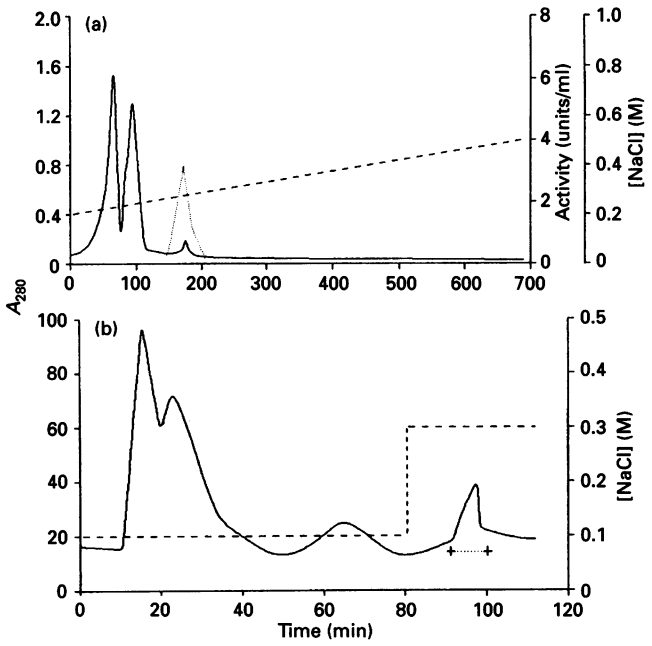

Figure 3 Chromatographic separation of culture fluid of *Streptoverticillium mobaraense* containing transglutaminase on Fractogel EMD SO_3^- (S-material) at pH 6.0 and room temperature

(**a**) Use of a 2.6 cm × 13 cm column. Elution of protein monitored at 280 nm (——) was achieved by a linear gradient of NaCl in 50 mM sodium phosphate buffer, pH 6.0 (------). The enzyme activity (·····) was measured with Cbz-L-glutaminylglycine. (**b**) Use of a 10 cm × 14 cm column. Elution of protein with stepwise gradient of 0.1 M and 0.3 M NaCl. +·····+, fractions with enzyme activity.

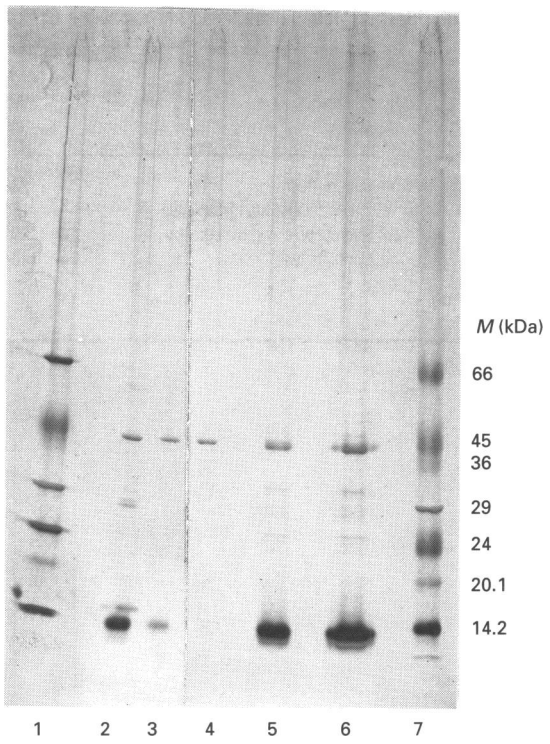

Figure 4 SDS/PAGE of transglutaminase fractions after different purification steps

Lanes 1 and 7, molecular-mass (*M*) markers; lane 2, sample after ion-exchange chromatography on Amberlite CG-50; lane 3, sample after ion-exchange chromatography on Amberlite CG-50 and hydrophobic chromatography on phenyl-Sepharose; lane 4, sample after ion-exchange chromatography on Fractogel EMD SO_3^-; lanes 5 and 6, culture fluid after centrifugation and filtration.

fold in a single step direct from the culture fluid. Very pure enzyme samples, showing only one band on electrophoresis, may be obtained by combining enzyme fractions comprising the maximum u.v. absorption peak.

Properties and storage stability of transglutaminase

After purification, transglutaminase of *Streptoverticillium mobaraense* has, with regard to its molecular mass, pI, pH optimum, temperature optimum and stability, the properties described above [12]. In our investigations, the Ca^{2+}-independent enzyme catalyses the acyl transfer of peptide-bound glutamine and not of asparagine. The reaction occurs considerably faster with bacterial transglutaminase than with the enzyme from guinea-pig liver homogenate (see also [16]). The K_m and $V_{max.}$ values of 12.2 mM and 17.4 μmol/min per mg of protein for the hydroxamate formation from Cbz-L-glutaminylglycin and hydroxylamine were calculated with variation of the dipeptide concentration between 1.0 and 36.0 mmol at pH 6.0 and 37 °C (correlation factor for nine values of 0.99996).

Bacterial transglutaminase is also a stable enzyme. Centrifuged and filtrated culture fluid could be stored over several months at −20 °C with an average loss of activity of 10%. Repeated thawing and refreezing did not significantly increase the enzyme loss. The purified and desalted enzyme was more stable, the more highly concentrated the sample was. Adding stabilizing additives such as glycerol or lyophilization of the desalted samples are not necessary. An aliquot could be stored over several days in a refrigerator without a measurable decrease in activity. The transglutaminase activity of an enzyme preparation with a concentration of 0.6 mg of protein/ml and a specific activity of 10 units/mg declined during storage at −20 °C with an approx. 40% loss in 6 months.

Conclusions

For the numerous potential applications of transglutaminase it is necessary to have simple and reproducible methods that allow the purification of the enzyme in good yields. A stable enzyme, in contrast with the guinea-pig liver transglutaminase, is produced by the micro-organism *Streptoverticillium mobaraense* which can be easily cultivated. The procedure proposed by Ando et al. [12] for the isolation of transglutaminase was, in our hands, not reproducible with sufficient effectiveness. Our variant, consisting of a modified ion-exchange chromatography on Amberlite CG-50 and hydrophobic chromatography, requires several days for the recovery of a transglutaminase preparation with sufficient purity. With the weak acid ion-exchange material one may additionally run the risk of a high enzyme loss by uncontrollable pH changes. The separation of the bound proteins was only moderate with both the ion-exchange and hydrophobic-chromatography methods (see Figures 1 and 2).

A more selective separation of transglutaminase from the other proteins, resulting in a very much better u.v. peak resolution (Figure 3), was successful when using the strong acid ion-exchange material Fractogel EMD SO_3^- whose ionic groups are

separated from the polymer matrix by long alkyl chains. By using low-pressure liquid-chromatography equipment we obtained enzyme preparations with high purity and in very good yields. Further purification by a second method may not be necessary. By suitable combining of the active fractions, only a single band is found on electrophoresis and silver staining.

The laboratory method could also be carried out with a small pilot plant. On the whole, it seems that the recovery of enzyme can be increased by up-scaling.

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