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High-throughput scintillation proximity assay for transglutaminase activity measurement

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Abstract

Members of the transglutaminase enzyme family are involved in a broad range of biological phenomena, including haemostasis, apoptosis, semen coagulation, skin formation, and wound healing. A new and rapid method for measurement of transglutaminase activity is described in this article. The enzyme links tritium-labeled putrescine to biotinylated oligoglutamine, and the tritiated peptide is bound to a streptavidin-coated microtiter plate permanently covered by a thin layer of scintillant. Only the radioisotope incorporated into the peptide substrate is close enough to the scintillant molecules for photons to be produced. The signal generation depends on the transglutaminase activity, and it can be detected by appropriate light-measuring instrumentation without separation steps. The assay is sensitive, specific, linear at concentrations of tissue transglutaminase between 0.05 and 1.6 mU/ml, and suitable for high-throughput measurements.

Keywords: Substrate; Oligoglutamine; Putrescine; Tritium; Inhibition; GTP

Transglutaminases (protein-glutamine:amine γ-glutamyltransferases, EC 2.3.2.13) represent a family of Ca^{2+}-dependent enzymes mediating covalent crosslinking reactions between specific peptide-bound γ-glutamyl residues and various primary amines, including ε-amino groups of peptide-bound lysyl residues. These enzymes stabilize biological structures via formation of covalent ε(γ-glutamyl)lysine isopeptide crosslinks resistant to proteases [1,2]. Primary amino groups can replace the lysyl residue, resulting in protein modifications affecting the biological activity of the target protein instead of polymer formation [3].

The transglutaminases are widely distributed enzymes existing in diverse organisms such as bacteria, plants, and animals with different cellular and extracellular localizations [4]. At least eight active enzymes in humans have been described [4,5]. Celiac disease [6], hypertrophic scarring [7], lamellar ichthyosis [8], and blood clotting abnormality [9] are the most important frequent disorders arising from improper function. The purified crosslinker transglutaminases are used as biocatalysts in commercial sectors related to the food, medical, cosmetic, and textile industries [10].

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There are differences in specificity and affinity for substrates among the members of the transglutaminase family. Extracellular as well as intracellular proteins have been shown to be Gln donor substrates for these enzymes [5,11]; however, most of the activity assays use *N*,*N*′-dimethylcasein. Previously described methods for the measurement of the activity have exploited the incorporation of radioactive (putrescine [12,13]), fluorescent (monodansyl-cadaverine [14,15]), and biotin-labeled (biotinamido-pentylamine [16,17]) amine substrates into *N*,*N*′-dimethylcasein. Other approaches for quantitation of enzyme activity have been to measure ammonia released during formation of the isopeptide bond [18–21] and to estimate the ε(γ-glutamyl)lysine crosslink [22]. Unfortunately, these techniques either are time-consuming and labor-intensive or are not sensitive enough for high-throughput studies on structure–function relationships of transglutaminases. These limitations prompted us to develop a versatile, simple, solid-phase method for rapid screening of activity using a scintillation proximity assay (SPA)[1] [23] with biotinylated oligoglutamine and [$^3H_{2,3}$]putrescine substrates. The designed biotinylated peptide can also be used for the detection of acyl acceptor substrates for transglutaminases.

Materials and methods

Materials

Fluorenylmethoxycarbonyl (Fmoc)-Gln-OH, Fmoc-Gln(trityl [Trt])-OH, Fmoc-Arg(2,2,5,7,8-pentamethyl chroman-6-sulfonyl [Pmc])-OH, and Wang resin were obtained from Novabiochem (Lucerne, Switzerland). Piperidine, *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxybenzotriazole, and methanol were of peptide synthesis grade and obtained from Merck (Darmstadt, Germany). The protein assay reagent was purchased from Bio-Rad (Richmond, CA, USA), and the Immobilon-P transfer membrane was obtained from Millipore (Bedford, MA, USA). [$^3H_{2,3}$]Putrescine dihydrochloride (35.74 and 32.60 Ci/mM) and 96-well streptavidin-coated FlashPlate microplates were obtained from Perkin-Elmer (Boston, MA, USA). 1,4-Dithiothreitol, streptavidin alkaline phosphatase, nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Coomassie brilliant blue (CBB), biotinamidocaproate *N*-hydroxysuccinimide ester, putrescine dihydrochloride, iodoacetamide, phenylmethylsulfonyl fluoride ($PhCH_2SO_2F$), hydrofluoric acid, αB-crystalline, dimethylformamide, GTP, Triton X-100, and *N*,*N*′-dimethylcasein all were purchased from Sigma (St. Louis, MO, USA). Purified guinea pig tissue transglutaminase was obtained from Sigma, according to which 1 U of activity catalyzes the formation of 1 μmol of hydroxamate per minute from *N*-α-CBZ-Gln-Gly and hydroxylamine at pH 6.0 at 37 °C [24]. The specific inhibitor peptide for tissue transglutaminases, the Boc-DON-Gln-Ile-Val-Ome, was obtained from N-Zyme BioTec (Darmstadt, Germany). Prestained SDS–PAGE molecular mass markers from Sigma were used (SDS 7B mixture, proteins with molecular masses of 180, 116, 84, 58, 48.5, 36.5, and 26.6 kDa). All other reagents and chemicals were of analytical grade.

Synthesis of biotinylated oligoglutamine

The 5Gln-Arg-4Gln decapeptide was produced by the Merrifield solid-phase method [25] using Fmoc, Trt, and Pmc protective groups on a 431A peptide synthesizer (Applied Biosystems, Foster City, CA, USA). The oligopeptide was N-terminally biotinylated on the resin with biotinamidocaproate *N*-hydroxysuccinimide ester in dimethylformamide. The final deprotection and cleavage from the resin were achieved by trifluoroacetic acid treatment. The crude product was purified by the 8A preparative HPLC system (Shimadzu, Kyoto, Japan) and lyophilized. The purity of the isolated peptide was estimated to be greater than 99% by analytical HPLC and MS (Finnigan MAT TSQ-700, San Jose, CA, USA).

Detection of acyl acceptor protein substrates for transglutaminases

Livers of 6-week-old wild-type mice were homogenized in 50 mM Tris–HCl (pH 7.5) containing 5 mM 1,4-dithiothreitol, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% (v/v) Triton X-100, and 2 mM $PhMeSO_2F$ using an electric Teflon homogenizer (MLW ER10) intermittently on ice for 5 min. Samples containing 100 μg protein were incubated at 37 °C for 30 min in 50 mM Tris–HCl (pH 7.5) in the presence of 5 mM 1,4-dithiothreitol, 5 mM Ca^{2+}, 2 mM $PhCH_2SO_2F$, and 0.1 mM biotinylated oligoglutamine in a total volume of 50 μl. Denaturing the solutions in a boiling water bath stopped the reaction. Samples containing 50 μg protein were run on 8% SDS–PAGE and blotted to Immobilon-P membrane, which was blocked with 2% nonfat dried milk, and were examined by streptavidin alkaline phosphatase using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate. Blanks without the peptide and control samples containing 10 mM iodoacetamide were also analyzed. Positive control experiments were carried out under the same conditions with 160 μU guinea pig tissue transglutaminase and 15 μg αB-crystalline. Molecular masses were determined

[1] *Abbreviations used:* SPA, scintillation proximity assay; Fmoc, fluorenylmethoxycarbonyl; Trt, trityl; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; CBB, Coomassie brilliant blue; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; TCA, trifluoroacetic acid.