UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos
Departamento de Ciências de Alimentos

Produção, purificação, caracterização e aplicação de transglutaminase de *Streptomyces* sp. CBMAI 837

Juliana Alves Macedo
Engenheira de Alimentos (FEA-UNICAMP)

Profa. Dra. Hélia Harumi Sato
Orientadora

Dra. Lara Durães Sette
Co-orientadora

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Maria Helena Andrade Santana

Roberto da Silva

Francisco Maugeri Filho

Eleonora Cano Carmona
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Banca Examinadora

Profa. Dra. Hélia Harumi Sato
(Orientadora) – DCA/FEA - UNICAMP

Profa. Dra. Maria Helena Andrade Santana
(Membro) – FEQ – UNICAMP

Prof. Dr. Roberto da Silva
(Membro) – IBILCE – UNESP

Prof. Dr. Francisco Maugeri Filho
(Membro) – DEA/FEA - UNICAMP

Profa. Dra. Eleonora Cano Carmona
(Membro) – Instituto de Biociências - UNESP

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"O coração do homem traça seu caminho, mas o Senhor lhe dirige os passos... Porque o Senhor dá sabedoria e da sua boca vem a inteligência e o entendimento".

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RESUMO GERAL

A transglutaminase (TGase) (EC 2.3.2.13; proteina-glutamina γ-glutaminiltransferase) é uma enzima capaz de catalisar reações de transferência de grupos acil utilizando resíduos de glutamina das ligações peptídicas de proteínas como doadores de grupos acil, e diversas aminas primárias como receptores. As ligações covalentes cruzadas entre inúmeras proteínas e peptídeos pela transglutaminase promovem mudanças nas propriedades de proteínas de alimentos. Por essa razão, a transglutaminase é amplamente utilizada nas indústrias de processamento de alimentos para o desenvolvimento de novos produtos e modificação de características como: viscosidade, capacidade emulsificante e valores nutricionais. Uma cepa de Actinomyceto, isolada de amostras de solo brasileiro, foi investigada taxonomicamente por uma combinação de técnicas moleculares e morfológicas, resultando na conclusão de que a cepa pertence ao gênero *Streptomyces* sp. A cepa, chamada de *Streptomyces* sp. CBMAI 837 produziu transglutaminase quando cultivada a 30°C por cinco dias, em agitador rotatório, no meio de fermentação otimizado, composto por: 0,2% KH_2PO_4, 0,1% $MgSO_4.7H_2O$, 2% farinha de soja, 2% amido de batata, 0,2% glicose, e 2% peptona, atingindo uma atividade enzimática de 1,37 $U.mL^{-1}$. A transglutaminase foi purificada cerca de 5 vezes através de duas passagens cromatográficas sucessivas em uma coluna de filtração em gel Sephadex G-75, com 17% de recuperação. A purificação da proteína foi comprovada por homogeneidade eletroforética em SDS-PAGE. A massa molar da TGase foi estimada em cerca de 45 kDa. A transglutaminase de *Streptomyces sp* CBMAI 837, tanto na forma bruta quanto

purificada, apresentou atividade enzimática ótima em pH 6,0-6,5, e em 35-40°C. Um segundo pico de atividade ótima foi observado em pH 10,0 na enzima no estado bruto. Ambas as formas da enzima foram estáveis na faixa de pH de 4,5 a 8,0 e até 45°C. A transglutaminase na forma bruta e purificada mostrou-se independente de íons cálcio, mas foi ativada na presença de K^+, Ba^{2+}, e Co^{2+}; e inibida por Cu^{2+} e Hg^2; o que sugere a presença de um grupo tiol no sítio ativo da enzima. A TGase purificada apresentou um K_m de 6,37 mM e um V_{max} de 1,70 U/mL, enquanto a enzima bruta apresentou K_m de 6,52 mM e um V_{max} de 1,35 U/mL para o substrato N-carbobenzoxi-L-glutaminil-glicina. A influência da transglutaminase de *Streptomyces* sp CBMAI 837 bruta, nas propriedades de géis ácidos de caseinato de sódio foi investigada, tendo como parâmetro géis preparados com a TGase comercial (Ajinomoto Inc.). Os géis com a enzima comercial tiveram um valor de módulo de elasticidade maior, porém, dependendo da concentração de proteína, estes foram menos deformáveis. Os géis com enzima bruta de *Streptomyces* sp. CBMAI 837 se mostraram muito mais rígidos e menos elásticos. Resultados da eletroforese indicaram que a enzima comercial promoveu a formação de polímeros de proteínas de massa molecular mais alta do que a enzima de *Streptomyces* sp. CBMAI 837. Os testes de microscopia eletrônica de varredura e da capacidade de retenção de água mostraram que características particulares de cada um dos géis poderiam estar associadas ao tipo específico de interação promovida por cada uma das amostras enzimáticas testadas.

Palavras chave: transglutaminase microbiana, *Streptomyces* sp., caracterização, purificação, gel de caseinato de sódio, taxonomia

GENERAL ABSTRACT

Transglutaminase (EC 2.3.2.13; protein-glutamine γ-glutaminyltransferase) is an enzyme that catalysis an acyl transfer reaction using protein or peptide-bond glutamine residues as acyl donors and several primary amines as receptors. The covalent cross-links between a number of proteins and peptides introduced by transglutaminase promote modification of the functional properties of the food proteins. Therefore, transglutaminase are widely used by food-processing industries for the purpose of new product development, modification of the product properties such as viscosity, emulsification foaming and nutritional values. An *actinomycete* strain, isolated from Brazilian soil, was taxonomically investigated using a combination of molecular and morphological based-methods, resulting on the conclusion that the strain belongs to the genus *Streptomyces* sp. The strain, named *Streptomyces* sp. CBMAI 837, produce transglutaminase when cultivated at 30°C for 5 days at 200 rpm in a rotatory shaker, on the optimized fermentation medium composed of 0.2% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, 2% soybean flour, 2% potato starch, 0.2% glucose, and 2% peptone, with a enzymatic activity of 1.37 $U.mL^{-1}$. The enzyme purification was performed by of two successive chromatographies on Sephadex G-75 columns with yields of 48% and 17%, respectively. The protein purification was successfully achieved to electrophoretical homogeneity on SDS-PAGE. The molecular mass of the MTGase was estimated to be about 45 kDa. The enzyme from *Streptomyces* sp., in both crude and pure forms, exhibited optimal activity in the 6.0-6.5 pH range and at 35-40°C. A second maximum of activity was observed at pH 10.0 on the crude *Streptomyces* sp. enzyme. Both forms of transglutaminase were stable over the pH range from 4.5 to 8.0 and up to 45°C. The activities of all the TGase samples were

independent of Ca^{+2} concentration, but they were elevated in the presence of K^{+}, Ba^{2+}, and Co^{2+} and inhibited by Cu^{2+} and Hg^{2+}, which suggests the presence of a thiol group in the TGase's active site. The purified enzyme presented K_m of 6.37 mM and V_{max} of 1.7 U/mL, while the crude enzyme demonstrated K_m of 6.52 mM and V_{max} of 1.35 U/mL. The influence of the transglutaminase on acid-gel properties was studied. Texture parameters showed that the commercial TGase (Ajinomoto Inc.) gels had greater values of elasticity modulus and could promote the formation of more elastic and soft food systems, while addition of the crude TGase of *Streptomyces* sp. CBMAI 837 to the gel led to the formation of more rigid and less elastic gels. The electrophoresis showed that the commercial TG enzyme in this system promoted higher molecular mass protein polymers than the enzyme from *Streptomyces* sp. CBMAI 837. Microscopy and water holding capacity (WHC) observations showed that all the gel characteristics could be associated to specific interactions promoted by each TGase tested.

Key words: microbial transglutaminase, *Streptomyces* sp., characterization, purification, caseinate gels, taxonomic

INTRODUÇÃO GERAL

A transglutaminase (TGase) é uma enzima caracterizada por sua capacidade de catalisar reações de transferência de grupos acil formando ligações cruzadas intra e intermoleculares entre proteínas, peptídeos e aminas primárias, principalmente através de ligações covalentes entre resíduos de glutamina e lisina. No mecanismo de reação catalisada pela TGase, o grupo tiol de uma cisteína da molécula enzimática ataca a cadeia lateral de um resíduo de glutamina acessível no substrato protéico, formando um complexo acil-enzima e amônia. No passo seguinte, uma amina primária substitui a enzima formando uma glutamina carboxamida modificada. Se a ligação protéica envolve a lisina, a ligação cruzada entre peptídeos, intra ou inter molecular, ocorre via N(γ-glutaminil)-L-lisina. Dessa forma, as reações com transglutaminase levam a formação de agregados protéicos irreversíveis. Esta enzima tem sido encontrada em mamíferos, moluscos, plantas e microrganismos. A transglutaminase parece estar envolvida em várias reações envolvendo proteínas; no entanto, a função mais estudada é sua participação no processo de coagulação sanguínea em mamíferos em que ocorre a formação de ligações cruzadas entre proteínas catalisada pelo fator XIIIa, uma forma ativada da enzima do plasma, e o estancamento do sangramento em ferimentos. Os estudos com microrganismos mostraram que linhagens do gênero *Streptomyces* têm a habilidade de produzir transglutaminase extracelular em grandes quantidades devido ao seu tipo de crescimento micelial no meio de cultura, que é característico do gênero. Entretanto, sua principal vantagem sobre as demais fontes da enzima é de produzir uma enzima cálcio independente, e de massa molecular relativamente baixa. Ambas as características são importantes para a aplicação industrial da enzima. Diversos estudos

demonstram que a transglutaminase microbiana é capaz de formar ligações cruzadas com a maioria das proteínas dos alimentos, tais como, caseínas, globulinas de soja, glúten, actina, miosina e proteínas dos ovos de forma tão eficiente quanto as TGases de mamíferos. Assim, a fermentação de *Streptomyces* constitui uma importante via biotecnológica de produção desta enzima. A modificação de proteínas dos alimentos pela transglutaminase pode levar à melhora da textura de vários produtos (lácteos, cárneos ou farináceos), pode ajudar na proteção e manutenção da lisina presente nos alimentos contra reações de degradação, viabiliza o encapsulamento de lipídeos e de materiais lipo-solúveis, forma filmes resistentes ao calor e à água, e fornece uma alternativa ao tratamento térmico para a gelificação de proteínas, melhora a elasticidade e a capacidade de retenção de água em vários produtos, é capaz de modificar a solubilidade e propriedades funcionais de proteínas, e pode produzir alimentos com proteínas de maior valor nutricional através da promoção de ligações cruzadas entre diferentes proteínas e amino ácidos complementares à dieta.

Este trabalho teve como principais objetivos selecionar uma linhagem produtora de transglutaminase e estudar a produção, purificação, caracterização e aplicação da enzima produzida por *Streptomyces* sp. Foi estudada a otimização da composição do meio de cultivo e as condições de fermentação do microrganismo em frascos agitados. Este trabalho traz uma significativa contribuição ao estudo da produção de transglutaminase por vias fermentativas, apresentando um novo microrganismo produtor da enzima, mudanças significativamente positivas no meio de cultivo normalmente apresentado na literatura, e novas informações sobre aplicação dessa enzima em géis de

caseinato. Este trabalho está apresentado na forma de capítulos baseados nos artigos científicos produzidos. A estrutura da tese foi organizada da seguinte forma:

O capítulo 1 trata de uma revisão bibliográfica sobre a transglutaminase e o estado da arte na sua produção por via fermentativa e sua aplicação industrial.

O capítulo 2 foi formado por duas publicações científicas, sendo a primeira uma publicação de resultados parciais, que descreveu o trabalho de isolamento e seleção dos microrganismos produtores de transglutaminase, e o estudo de otimização dos componentes do meio de cultivo do microrganismo em frascos agitados. Nesse trabalho, ficou registrado o isolamento de uma nova linhagem produtora da enzima, proveniente de amostras de solo brasileiro. O segundo trabalho apresentou o estudo da otimização das condições de fermentação da linhagem *Streptomyces* sp. para produção da enzima. Esse trabalho englobou os resultados de otimização da composição do meio de cultivo, apresentados na primeira publicação, e também o estudo de otimização de outros parâmetros importantes para a fermentação em frascos agitados, como a temperatura, agitação e estudo cinético da produção. O estudo da produção da enzima foi realizado empregando a metodologia de superfície de resposta, avaliando os principais parâmetros de fermentação da linhagem de *Streptomyces* sp. CBMAI 837 em escala de laboratório.

No capítulo 3 foram abordados aspectos da purificação da transglutaminase, que foi realizada através da aplicação de uma solução da enzima bruta liofilizada por duas vezes sucessivas em uma coluna de filtração em gel Sephadex G-75. O fator de purificação obtido foi de 5,0 com 17% de recuperação. A purificação da proteína foi comprovada por homogeneidade eletroforética em SDS-PAGE.

O capítulo também descreve a caracterização bioquímica da enzima bruta, purificada e também da enzima comercial Activa® TG-BP (Ajinomoto Inc.). Trata-se da avaliação dos aspectos da enzima relacionados a sua atividade catalítica, como por exemplo: temperatura ótima de atividade e estabilidade, efeito do pH ótimo de atividade e estabilidade da enzima, estudo de alguns ativadores e inibidores da atividade da transglutaminase em estado bruto e purificado. Os resultados foram comparandos com as características obtidas para a enzima comercial.

O capítulo 4 tratou da aplicação da enzima transglutaminase em géis de caseinato de sódio. A enzima bruta foi utilizada nesse estudo, assim como a enzima comercial (Activa® TG-BP da Ajinomoto Inc.), de forma que as características conferidas pela ação das enzimas na rede protéica durante a formação dos géis pudessem ser comparadas entre si, e com o gel padrão, que não recebeu tratamento enzimático. Esses estudos de melhoramento da textura e da capacidade de retenção de água, entre outras características, têm se mostrado importantes para a consolidação da transglutaminase como uma solução tecnológica interessante para a melhoria da qualidade de produtos lácteos na indústria de alimentos moderna.

O quinto, e último capítulo, descreveu o esforço realizado a fim de se investigar a taxonomia da linhagem isolada. A primeira etapa de identificação foi baseada nos métodos de isolamento e amplificação da sequência parcial de DNA ribossômico 16S. Essa informação foi comparada com a base de dados do Genbank, e os resultados mostraram que a linhagem de actinomiceto P20 pertence ao gênero *Streptomyces* sp. A análise da árvore filogenética indicou grande semelhança entre a linhagem de *Streptomyces* sp P20 com as linhagens S. *caniferus, S. platensis e S. hygroscopicus.*

Técnicas de identificação morfológicas e fisiológicas aplicadas à linhagem *Streptomyces* sp. P20 corroboraram sua classificação como gênero *Streptomyces*, e excluíram a possibilidade de pertencer à espécie *Streptomyces hygroscopicus*. Porém, apesar das semelhanças com o *S. caniferus* e com *S. platensis*, toda informação obtida ainda não é conclusiva para determinar a espécie, e também não permite estabelecermos uma nova espécie de *Streptomyces*. Estudos mais aprofundados de taxonomia serão realizados em parceria com pesquisadores do Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - DSMZ (German Collection of Microorganisms and Cell Cultures).

CAPÍTULO 1. PROPRIEDADES E APLICAÇÕES DA TRANSGLUTAMINASE MICROBIANA EM ALIMENTOS

Juliana Alves Macedo[1] e Hélia Harumi Sato[1]

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[1] Departamento de Ciência de Alimentos- Faculdade de Engenharia de Alimentos- UNICAMP- 13083-862- Campinas -SP- Brasil.

RESUMO

As propriedades e aplicações da transglutaminase (TGase) chamada de TGase microbiana (MTGase) descritas neste trabalho, se referem à enzima proveniente de variantes de *Streptomyces mobaraensis*. A MTGase mostrou ser capaz de fazer ligações cruzadas em grande parte das proteínas de alimentos, tais como caseínas, globulinas de soja, glúten, actinas, miosinas e proteínas de ovos, de forma tão eficiente quanto as TGases de origem animal obtidas de mamíferos. Entretanto, ao contrário de muitas outras TGases, a MTGase é uma enzima cálcio-independente e possui uma massa molecular relativamente baixo. Estas duas características são vantajosas no que diz respeito à aplicação industrial desta enzima. Muitos estudos demonstram o grande potencial de aplicação das MTGases no processamento de alimentos e em outras áreas. A MTGase vem sendo estudada com intuito de modificação de alimentos protéicos processados, implementando propriedades funcionais a produtos, ou como mostram alguns estudos, melhorando características como sabor, aroma e textura. Além disso, o uso desta enzima mostrou aumentar o tempo de prateleira de determinados produtos e reduzir o potencial alergênico de outros.

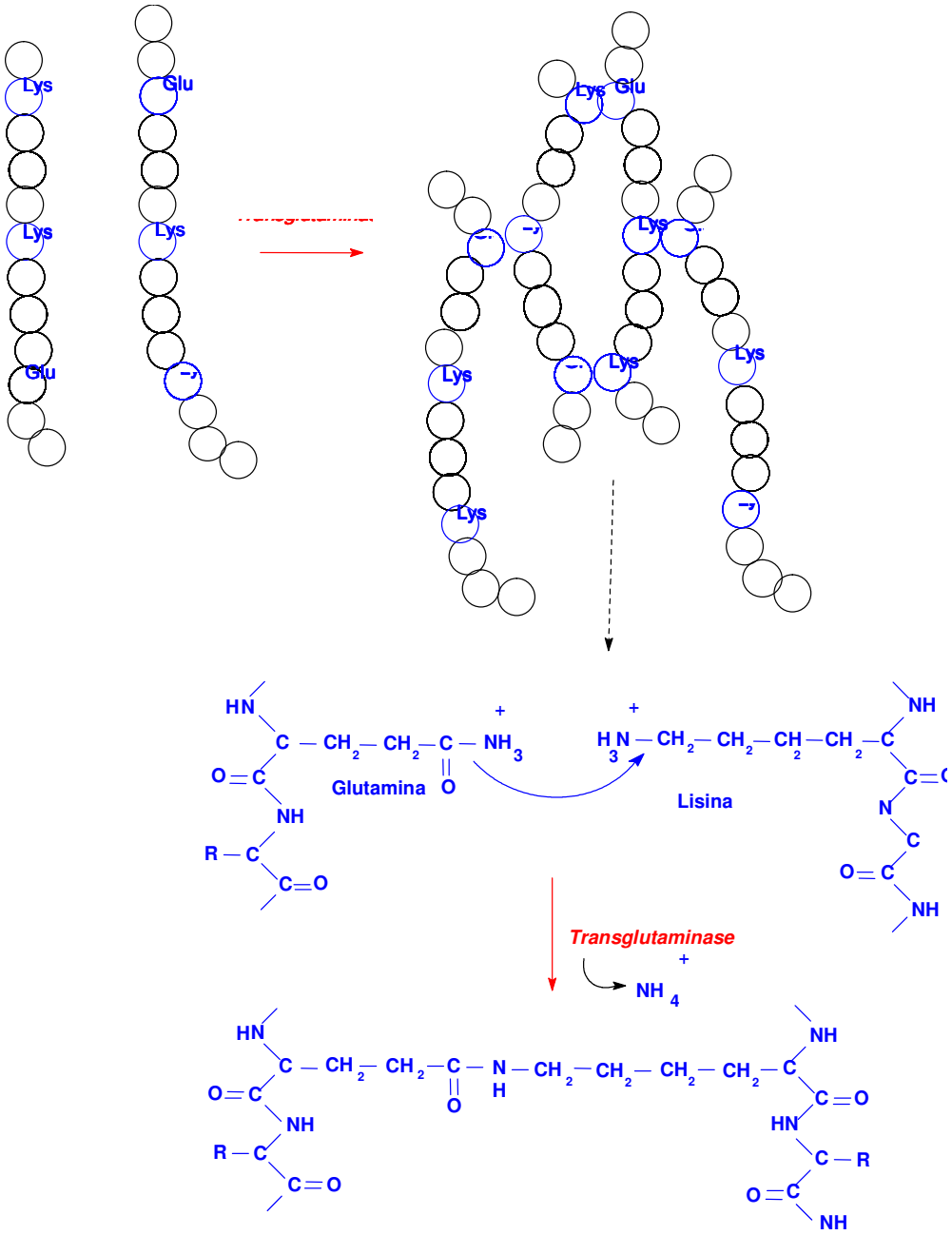
PALAVRAS-CHAVE: Transglutaminase; propriedades funcionais e nutricionais; aplicações industriais

INTRODUÇÃO

A transglutaminase (TGase) (EC 2.3.2.13) é uma enzima capaz de catalisar reações de transferência de grupos acil formando ligações cruzadas intra e intermoleculares em proteínas, peptídeos e várias aminas primárias, principalmente através de ligações covalentes entre resíduos de glutamina e lisina (Nonaka et el., 1989).

No mecanismo de reação catalisada pela TGase, o grupo tiol de uma cisteína ataca a cadeia lateral de um resíduo de glutamina acessível no substrato protéico, formando um complexo acil-enzima e amônia. No passo seguinte, uma amina primária entra no lugar da enzima formando uma glutamina carboxamida modificada. Se a ligação protéica envolve a lisina, a ligação cruzada entre peptídeos, intra ou inter molecular, ocorre via N(γ-glutaminil) L-lisina. Dessa forma, as reações com transglutaminase levam a formação de agregados protéicos irreversíveis, sendo muito importante a regulação da ação desta enzima nos organismos (Pasternack et al., 1998). As Figuras 1.1 (a), (b) e (c) ilustram o modo de ação da transglutaminase.

(a)


Lys
Glu
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Lys
Lys
Lys
Glutamina
Lisina
Transglutaminase
NH 4

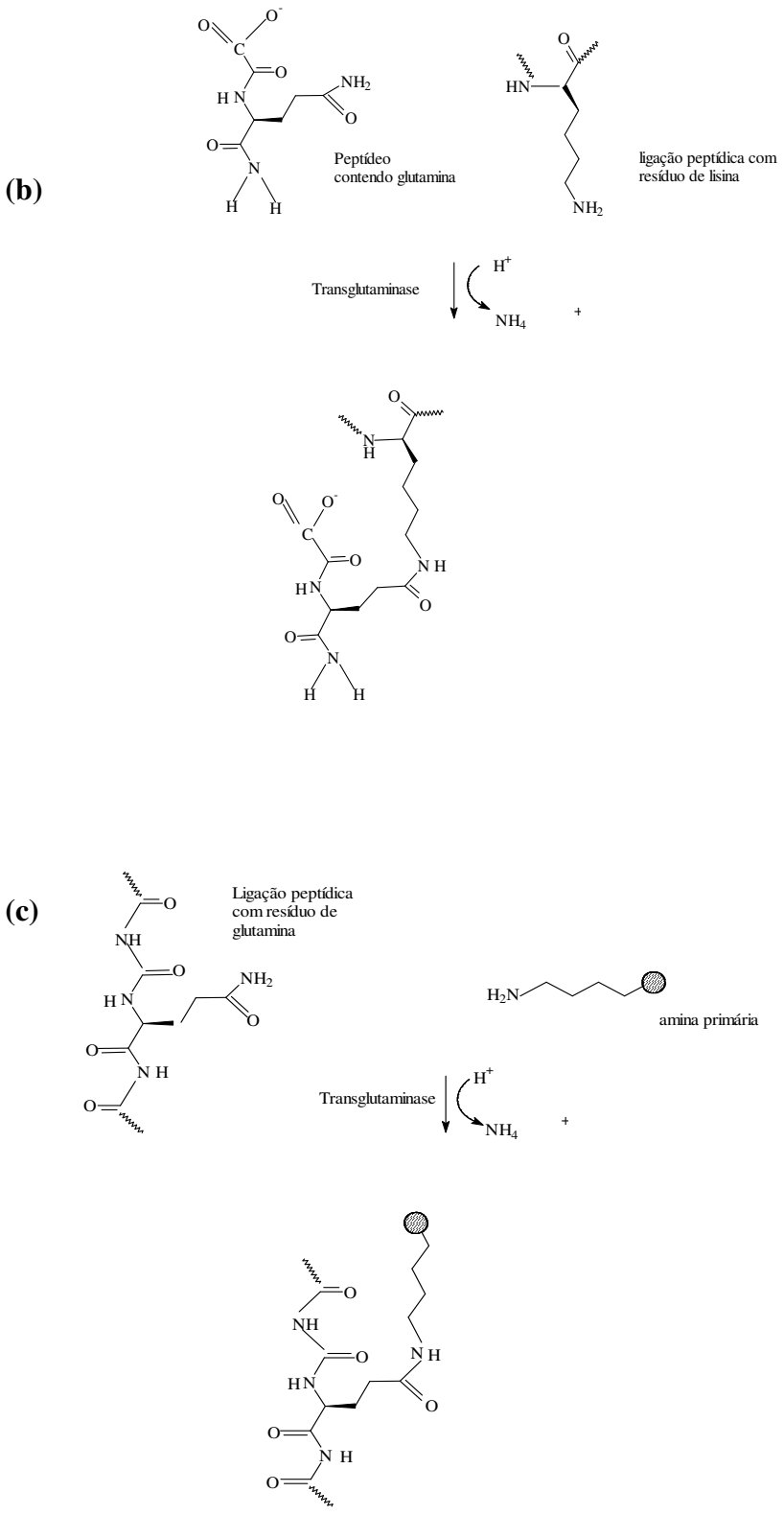

Figura 1.1. Modo de ação da transglutaminase. a) Modo de ação geral da enzima. b) Ligações cruzadas entre resíduos de lisina e glutamina em proteínas ou peptídeos. c) Reação entre resíduo de glutamina e aminas primárias

Esta enzima tem sido encontrada em mamíferos, vertebrados, invertebrados, moluscos, plantas e microrganismos (Pasternack et al., 1998). A transglutaminase parece estar envolvida em inúmeras reações envolvendo proteínas; no entanto, a função mais estudada é sua participação no processo de coagulação sanguínea em mamíferos em que ocorre a formação de ligações cruzadas entre proteínas catalisada pelo fator XIIIa, uma forma ativada da TGase do plasma, e o estancamento do sangramento em ferimentos (Chung et al., 1974 e Hornyak et al., 1989). Outro exemplo é o fenômeno interessante de "suwari" ou endurecimento da pasta de proteína de peixe moído a baixa temperatura, pela formação de ligações cruzadas na fabricação do kamaboko, devido a TGase presente naturalmente no peixe (Seki et al., 1990).

Recentemente a transglutaminase microbiana adquiriu importância devido às inúmeras aplicações possíveis em alimentos, tornando importante buscar o aumento da disponibilidade e redução de custos de produção.

Os estudos baseados na seleção de milhares de microrganismos mostram que linhagens do gênero *Streptoverticillium* têm a habilidade de produzir a transglutaminase em grandes quantidades devido ao tipo de crescimento micelial no meio de cultura, que é característico do gênero (Yan et al., 2005). Entretanto, sua principal vantagem sobre todas as outras fontes da enzima é de produzir uma enzima cálcio independente, e de massa molecular relativamente baixa. Ambas as características são importantes para a aplicação industrial da enzima (Yokoyama et al., 2004).

Diversos estudos demonstram que a TGase produzida por microrganismos é capaz de formar ligações cruzadas com a maioria das proteínas dos alimentos, tais como,

caseínas, globulinas de soja, glúten, actina, miosina e proteínas dos ovos de forma tão eficiente quanto as TGase de mamíferos (Yokoyama et al., 2004).

Estudos das possibilidades de modificação de proteínas pela TGase

No início dos anos 80, a possibilidade de modificação do comportamento de caseínas de leite, e globulinas de soja foi demonstrada utilizando a TGase proveniente do soro de porquinhos da índia (Ikura et al., 1992) ou do plasma bovino (Kurth e Rogers, 1984). Nestes estudos, as ligações cruzadas entre proteínas de diferentes origens foram identificadas; assim como a incorporação de aminoácidos ou peptídeos para suprir deficiências nutricionais.

Neste mesmo período, grupos de pesquisadores pertencentes ao "Ajinomoto Institute of Life Sciences" realizaram uma série de estudos sobre a viabilidade da aplicação da enzima do soro de porquinhos da índia, na modificação industrial de proteínas de alimentos. Estes estudos (Motoki et al., 1983, 1984, 1987a, 1989; Nio e Motoki, 1986; Nio et al., 1985, 1986) incluíram proteínas diversas e actomiosina de carne bovina, porco, frango e peixe, como substratos a serem gelificados. Subseqüentemente, os estudos evoluíram para melhorias na solubilidade, capacidade de retenção de água e estabilidade térmica das proteínas de alimentos. As proteínas presentes em emulsões do tipo óleo em água também foram gelificados pela TGase, o que significa que a enzima se mostrou eficiente em seus propósitos.

Alguns destes estudos mostraram ser possível criar um filme transparente, resistente à água e de digestão lenta a partir de soluções de proteínas aspergidas em superfícies planas. Com base em todos estes resultados, a TGase foi considerada potencialmente interessante para a criação de proteínas com novas propriedades. Porém,

a fonte de origem animal da enzima tornava inviável sua produção para utilização em larga escala pela indústria de alimentos.

Obtenção de transglutaminase para aplicação industrial

De forma geral, existem três maneiras de buscar o desenvolvimento da produção de uma fonte de transglutaminase para fins industriais. A primeira é extrair e purificar a enzima de tecidos e fluidos corporais de animais usados na alimentação humana, tais como: bovinos, suínos ou pescados. Na Europa, o fator XIII, um tipo de TGase, é extraído comercialmente de sangue de gado e de suínos de abate (Yamauchi et al., 1991). A enzima de sangue é muito pouco utilizada na fabricação de alimentos, uma vez que possui uma coloração vermelha que prejudica a coloração dos produtos. Além disso, essa enzima precisa de uma protease específica, chamada trombina, para sua ativação.

A segunda forma de obter a enzima é através de manipulação genética utilizando microrganismos hospedeiros, como a *E. coli*, *Bacillus*, leveduras ou *Aspergillus*. Muitos pesquisadores têm tentado obter a TGase em grandes quantidades a baixos custos (Bishop, 1990; Ikura et al., 1998; Yasueda et al., 1995). Recentemente, obteve-se sucesso com uma secreção eficiente de TGase de *Streptomyces* pelo *Corynebacterium glutamicum,* uma bactéria muito utilizada na produção industrial de aminoácidos, tais como lisina e glutamato (Yokoyama et al., 2004). Entretanto, nenhuma dessas enzimas têm sido comercializadas; em grande parte por problemas de falta de aceitação do mercado, devido ao fato de serem oriundas de microrganismos geneticamente modificados. A terceira forma de obter a enzima é realizar um "screening" por um microrganismo produtor de TGase. Com um microrganismo apropriado, seria possível produzir TGase em grande quantidade pela tecnologia tradicional de fermentação.

Microrganismos produtores de transglutaminase

A produção de TGase de microrganismos foi relatada no final da década de 80. Ando e Motoki entre outros pesquisadores, exploraram a possibilidade de produzir transglutaminase de microrganismos (Ando et al., 1989; Motoki et al., 1989). Realizou-se um "screening" com mais de 5000 cepas isoladas de solos coletados em diversas localidades. Dentre estas cepas, o *Streptoverticillium* S-8112 demonstrou ter a capacidade de produzir transglutaminase (Ando et al., 1989). Outras cepas de *Streptoverticillium*, tais como *S. griseocarneum*, *S. cinnamoneum* subsp. c*innamoneum* e *S. mobaraense*, também possuíam a capacidade de produzir a MTGase (Motoki, et al., 1989). Existem, na realidade, poucos estudos sobre TGase produzida por microrganismos. A atividade de transglutaminase foi encontrada nos gêneros *Physarum polycephalum*, *Candida albicans*, *Bacillus subtilis*, *Bortedella pertussis* e *E. coli*, porém, nem sempre a enzima era extracelular ou excretada em quantidades significativas como nas cepas de *Streptoverticillium* (Klein et al., 1992; Lin et al., 2003; Soares et al., 2003).

As enzimas produzidas por *Streptoverticillium* sp. S-8112*, S. mobaraense, P. polycephalum, Sv. ladakanum e Sv. cinnamoneum* CBS 683.68 já foram purificadas e caracterizadas. Os genes para produção de TGase de *Streptoverticillium* sp. S-8112, *Sv. cinnamoneum* CBS 683.68, *Sv. mobaraense* DSMZ e *B. subtilis* foram clonados e seqüenciados (Klein et al., 1992; Lin et al., 2003; Soares et al., 2003).

Produção de transglutaminase microbiana (MTGase)

O procedimento de fermentação para produção de MTGase é basicamente o mesmo para os diversos microrganismos mencionados (Ando et al., 1992; Ando et al., 1989; Motoki et al., 1989). Glicose, sacarose, amido, glicerina e dextrina podem ser

usados como fontes de carbono. Como fonte de nitrogênio, podem ser usadas tanto fontes inorgânicas, como as orgânicas: NH_4NO_3, $(NH_4)_2SO_4$, uréia, $NaNO_3$, NH_4Cl, soja, arroz, trigo ou farinha de trigo, peptona, extrato de carne, caseína, aminoácidos ou extrato de malte. Os minerais necessários e microelementos são: fosfato, magnésio, potássio, ferro, cobre, zinco e vitaminas; podem ser adicionados, se necessário, surfactantes não iônicos, e antiespumante.

O microrganismo realiza crescimento aeróbico, e por isso aeração e agitação são necessários. A temperatura ótima de crescimento e formação da enzima está entre 25°C e 35°C, e o tempo de fermentação depende das condições da cultura e é determinado pela maior atividade de transglutaminase atingida, normalmente entre 2-4 dias.

A MTGase, em quase todos os casos, é uma enzima extracelular, e fica dissolvida no caldo de fermentação, e por isso pode ser recuperada pela separação do material sólido do caldo de fermentação. Os métodos normalmente utilizados para a purificação de enzimas podem ser aplicados na MTGase. Dessa forma, etanol, acetona, álcool isopropílico e outros solventes orgânicos podem ser utilizados no processo de "downstream" da enzima. A precipitação com sulfato de amônio e cloreto de sódio, diálise, ultrafiltração, cromatografia por afinidade, filtração em gel e método do ponto isoelétrico podem ser utilizados para purificação da enzima. Uma boa combinação dos métodos mencionados pode tornar a purificação eficiente e aumentar a taxa de recuperação da enzima.

A enzima obtida pode então ser misturada com estabilizantes tais como diversos sais, açúcares, proteínas, lipídeos e surfactantes (Sakamoto et al., 1992).

Exemplos de fermentações e purificações foram descritos por Ando e Motoki, entre outros, em seus trabalhos (Ando et al., 1992; Ando et al., 1989; Motoki et al., 1989). Os microrganismos foram pré-cultivados em um meio composto de 0,2% de peptona, 0,5% glicose, 0,2% K_2HPO_4, 0,1% $MgSO_4$ em pH 7,0. A cepa foi então inoculada em 100 mL de meio de cultura em frascos Sagakuchi de 500 mL e cultivadas a 30°C por 48h. O caldo de fermentação obtido foi adicionado a 10 L de meio de cultura fresco (pH 7,0) composto de 0,2% de peptona, 2,0% de amido solúvel, 0,2% K_2HPO_4, 0,1% de $MgSO_4$, 0,2% de extrato de levedura e 0,05% antiespumante, e cultivados por 3 dias a 30 ºC sob aeração (10 L/min) e agitação (250 rpm).

Foram descritas atividades de transglutaminase de 0,28 U/mL até 2,5 U/mL, dependendo do microrganismo utilizado. A massa celular foi separada por centrifugação a 3000 rpm. O líquido claro obtido foi concentrado por membrana de ultrafiltração e tratado em uma coluna de Amberlite CG-50 que foi equilibrada com tampão fosfato de sódio 0,05M (pH 6,5). A coluna foi lavada com o mesmo tampão e as frações ativas foram reunidas. As frações ativas reunidas foram tratadas novamente na coluna Amberlite CG-50 sob as mesmas condições. A fração ativa foi diluída para reduzir a condutividade e então passou pela coluna Blue Sepharose. Depois deste tratamento, a enzima estava purificada 174 vezes. O total da recuperação da atividade da transglutaminase foi de cerca de 42%.

Recentemente, uma modificação no processo de "downstream" para purificação da MTGase foi desenvolvida. Depois da fermentação, o caldo foi centrifugado e filtrado. A enzima foi separada diretamente com um trocador catiônico forte, em uma única etapa.

De acordo com os autores, este método é simples, rápido, e a recuperação da transglutaminase é de cerca de 40% (Gerber et al., 1994).

Características físico-químicas da MTGase

As características físico-químicas da MTGase foram descritas em alguns trabalhos (Ando et al., 1989; Kanaji et al., 1993; Nonaka et al., 1989). A massa molecular da enzima foi estimada em aproximadamente 38 kDa, tanto por eletroforese SDS-poliacrilamida (SDS-PAGE), quanto por cromatografia de gel-permeação (Ando et al., 1989) e o ponto isoelétrico (pI) em 9,0. O sequenciamento de proteínas, tanto pelo método automatizado de Edman, quanto por espectrometria de massa, revelou que sua estrutura primária tem 331 aminoácidos (Kanaji et al., 1993). O sequênciamento do cDNA do microrganismo produtor forneceu resultados consistentes, a MTGase possui um único resíduo de cisteína e massa molecular estimada em 37.842 Da, próximo do valor obtido experimentalmente de 38.000 Da (Washizu et al., 1994). Desta forma, a MTGase é uma proteína monomérica e simples (não uma lipoproteína ou glicoproteína), embora tenha dois sítios potenciais de glicosilação (-Thr-Xxx-Asn-).

Propriedades enzimáticas da MTGase

O pH ótimo para a atividade da MTGase se mostrou entre 5 e 8, entretanto a enzima apresentou alguma atividade em uma faixa de pH de 4 a 9. A enzima também foi considerada estável em uma ampla faixa de pH (Ando et al., 1989).

A temperatura ótima para atividade enzimática foi de 55°C (por 10min a pH 6,0); ela manteve toda sua atividade quando mantida por 10 min a 40°C, porém perdeu atividade em apenas alguns minutos a 70°C. A enzima ainda estava ativa a 10°C, e

manteve atividade residual até perto da temperatura de congelamento. No que diz respeito à especificidade quanto ao substrato, a maioria dos alimentos ricos em proteínas pode ter ligações cruzadas em seus grupos protéicos. Alimentos como globulinas de legumes, glúten de trigo, gema de ovo e albuminas, actinas, miosinas, fibrinas, caseínas de leite, alfa-lactoalbuminas e beta-lactoglobulina, assim como muitas outras albuminas, foram testadas e tornaram-se substrato para ligações cruzadas feitas pela MTGase (Kang et al., 1994; Nonaka et al., 1997; Nonaka et al., 1992; Seguro et al., 1995a).

A Tabela 1.1 ilustra as características bioquímicas de algumas transglutaminases descritas na literatura.

Tabela 1.1. Características bioquímicas de algumas transglutaminases microbianas extracelulares

Microrganismo	pH ótimo de atividade	Temperatura ótima de atividade (°C)	pH de estabilidade	Estabilidade Térmica (°C)	Referência
***Streptoverticillium* sp. S-8112**	6 – 7	_	5 – 9	_	(Ando et al., 1987)
Sv.mobaraense	6,5 - 7,0	28 – 32	5 – 8	_	(Meiying et al., 2002)
Sv. ladakanum	5-6	40	5 – 7	Até 50-55	(Ho et al., 2000)
Bacillus circulans	7,0	47	6 – 8	_	(Soares et al., 2003)

TGases, incluindo as bem caracterizadas enzimas provenientes de soro de porquinho da índia, em geral são extremamente dependentes da presença de íons Ca^{+2}. Entretanto, as MTGases são totalmente independentes da presença de íons Ca^{+2}, e sob esse ponto de vista trata-se de enzimas diferentes das presentes nos mamíferos (Aechlimann e Paulsson, 1994). Essa propriedade é muito importante na aplicação da enzima para modificação de proteínas de alimentos, tais como caseínas de leite,

globulinas de soja e miosinas, uma vez que estas são bastante sensíveis e precipitam com facilidade na presença de íons Ca^{+2}. A sensibilidade da MTGase a outros cátions na ausência de agentes redutores foi investigada. Cu^{+2}, Zn^{+2}, Pb^{+2} e Li^{+} se mostraram fortes inibidores da enzima microbiana. Como se espera que metais pesados, tais como Cu^{+2}, Zn^{+2} e Pb^{+2}, se liguem ao grupo tiol do único resíduo de cisteína presente na enzima, trata-se de um indício de que esse resíduo de cisteína faça parte do sítio ativo da enzima.

As MTGases são capazes de gelificar soluções concentradas de proteínas de soja, leite, carne bovina, de porco, frango, peixe e miosinas de forma muito semelhante à TGase de soro de porquinho da índia (Kang et al., 1994; Neilsen, 1995; Nonaka et al., 1997; Nonaka et al., 1992; Seguro et al., 1995a). Quando gelificada, a globulina de soja torna-se mais firme se aquecida a 100°C por 15 min, adquirindo novas propriedades de gel. Caseínas de leite também são gelificadas pela MTGase sem necessidade de aquecimento e o gel, assim formado, não mais se dissocia quando atinge 100°C.

Proteínas diferentes podem ter ligações covalentes cruzadas feitas pela MTGase para produzir combinações com novas funcionalidades. Um exemplo seria a conjugação de caseínas do leite, ou globulinas da soja com ovomucina, gerando uma glicoproteína branca de ovo, cujo poder emulsificante é maior que o de qualquer uma das proteínas originais (Kato et al., 1991). Em outro trabalho, a combinação de caseína e gelatina gerou uma nova proteína altamente solúvel em pH ácido (Neilsen, 1995).

A MTGase é capaz de incorporar aminoácidos e peptídeos com ligações covalentes em substratos protéicos. Em aplicações práticas, todos os aminoácidos, com exceção da lisina, deveriam ter seus grupos alfa-carboxil amidados, ou esterificados ou ainda descarboxilados para eliminar suas cargas negativas. A lisina, cujo grupo ε-amino é

uma amina primária, é um bom substrato para a MTGase. Nestas reações, a proteína atua como um doador de acil, enquanto os aminoácidos, incluindo a lisina, atuam como um acil receptor.

Os peptídeos contendo, tanto a lisina quanto a glutamina, são capazes de servir de substrato sem sofrer nenhuma modificação. Os peptídeos com lisina atuam como receptores de acil, enquanto as proteínas são os doadores de acil; já os peptídeos com glutamina atuam como acil doadores enquanto as proteínas envolvidas são receptores de acil. Desta forma, lisilmetioninas podem ser incorporadas em caseínas para combater a deficiência de metionina, assim como lisilarginina pode ser incorporada em caseínas também, para combater a deficiência de arginina.

Aplicações da MTGase no processamento de alimentos

Na indústria de produtos cárneos, a possibilidade de obter, a partir de retalhos, pedaços grandes de carne reestruturada na forma de bifes de carne bovina ou filés de peixe tem se demonstrado muito interessante. Estudos mostram que um novo sistema de unir pedaços de carne utilizando a MTGase e caseinato tem muita eficácia. O mais importante é que esta ligação não depende da aplicação de cloreto de sódio ou de fosfatos, criando produtos mais saudáveis em função dos baixos teores de sais de sódio (Wilson, 1992; Yokoyama et al., 2004).

O uso da MTGase na reestruturação de recortes de carne sem valor para obtenção de peças de carne reestruturada de melhor aparência se mostrou muito eficaz, porém, o surpreendente foi a melhora na textura e sabor da peça, que pode atingir maior valor de mercado (Kim et al., 1993).

A MTGase se mostrou capaz de gelificar soluções concentradas de proteínas como: proteínas de soja, do leite, de carne bovina, suína e de peixe. Além disso, pôde incorporar aminoácidos ou peptídeos em proteínas, através de ligações covalentes. Este tipo de reação é importante, pois pode aumentar o valor nutricional de alimentos, uma vez que os aminoácidos incorporados com ligações covalentes se comportam no organismo da mesma forma que os aminoácidos endógenos (Yokoyama et al., 2004). Um bom exemplo seria a inclusão de metionina e lisina em proteínas pobres nestes aminoácidos, tais como caseínas de leite e proteínas de soja, através de reações catalisadas pela TGase.

Na área dos lacticínios, a formação de um gel resistente ao calor, a partir de caseína e da MTGase, promete a criação de iogurtes mais estáveis, evitando a sinerese. A enzima também viabiliza produtos como sorvetes e queijos com baixo teor de gordura, mais estáveis e com melhor textura (Yokoyama et al., 2004).

Outros produtos alternativos, como os similares aos laticínios feitos a base de soja (queijos e iogurtes de soja) também são um grande campo de atuação para as transglutaminases. Por exemplo, o tofu, um produto típico, feito da coagulação de extrato de soja com íons cálcio e magnésio, tem uma vida de prateleira muito curta. Entretanto, a adição de MTGase tem permitido a manutenção do tofu pasteurizado por muito mais tempo, tornando sua textura mais firme (Kwan e Easa, 2003).

A aplicação de MTGase foi testada também em macarrões instantâneos e outras massas, e tem tido sucesso na preservação da textura da massa pós cozimento, mesmo quando a farinha de trigo usada era de qualidade inferior. Foi sugerido também, que o

volume de crescimento de muitos pães poderia ser aumentado com o uso da enzima (Yokoyama et al., 2004).

Foi desenvolvido um método para reduzir o potencial alergênico de algumas proteínas de alimentos ou de peptídeos. Alfa-caseína (23kDa) foi tratada com transglutaminase a 25°C por 20h em água para produzir caseína com ligações cruzadas (aproximadamente 90kDa), que é menos alergênica (Yamauchi et al., 1991).

Um material que promove a absorção de minerais pelo corpo humano foi desenvolvido por Noguchi e outros pesquisadores (Noguchi et al., 1992). Ele é preparado pela desaminação de caseína através de um tratamento com transglutaminase. O material resultante promove a absorção de minerais no intestino e pode ser usado na indústria de alimentos e farmacêutica em suplementos minerais para adultos e crianças. A caseína é solúvel em pH neutro e levemente ácido, podendo manter os minerais solúveis no intestino.

Perspectivas

Em todo o mundo, a pesquisa por novas fontes de proteínas para alimentação humana ou por formas de aumentar a disponibilidade das fontes existentes, é uma prioridade. Nos países em desenvolvimento muitas pessoas ainda sofrem com a subnutrição e muitos esforços são feitos para produzir fontes de proteínas alternativas à carne aceitáveis pela população e que possam resolver os problemas de deficiência protéica (Steinkraus, 1994). Por outro lado, em países desenvolvidos, além das preocupações com os problemas de saúde relacionados à obesidade, as pessoas estão muito cientes do elevado custo causado pela superprodução de gado. Quando são defrontados com um novo produto, os consumidores se tornam muito sensíveis a aspectos

tais como o aroma, valor nutricional, aparência, vida de prateleira, e palatabilidade. Considerando todos estes aspectos, proteínas modificadas com enzimas, especialmente a transglutaminase microbiana, são uma das mais promissoras alternativas para produção de novos alimentos proteicos; principalmente devido sua potencialidade de ser produzida em grandes quantidades por fermentação de substratos de baixo custo.

No que diz respeito a produção de MTGase por microrganismos, uma vantagem é de independer de fatores como condições climáticas e regionais, além de seu custo bastante razoável. Porém, ainda é muito importante desenvolver os estudos da fermentação e recuperação da enzima a fim de reduzir ainda mais seus custos. A modificação genética de espécies por engenharia genética é uma das alternativas para estudos futuros. Entretanto, existe uma tendência muito forte em muitos países de não aprovar a aplicação de microrganismos geneticamente modificados em alimentos ou na produção de ingredientes para alimentos (Jank, 1995). Dessa forma, melhoramentos no processo tecnológico, adoção de nova tecnologia de fermentação ou combinações de ambos os fatores oferecem perspectivas promissoras na área.

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CAPÍTULO 2. OPTIMIZATION STUDIES FOR THE PRODUCTION OF MICROBIAL TRANSGLUTAMINASE FROM A NEWLY ISOLATED STRAIN OF *STREPTOMYCES* SP

Juliana Alves Macedo[2], Lara Durães Sette[1], Hélia Harumi Sato[2]

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[1] Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), Microbial Resource Division, CPQBA/UNICAMP, P.O. Box 6171, CEP 13081-970, SP, Brazil.

[2] Food Science Department, Faculty of Food Engineering, UNIVERSIDADE ESTADUAL DE CAMPINAS (UNICAMP) P.O. Box 6121, CEP 13083-862, SP, Brazil.

ABSTRACT

Covalent cross-links between a number of proteins and peptides explain why transglutaminase may be widely used by food-processing industries. The objective of this work was the optimization of the fermentation process to produce transglutaminase from a new microbial source, the *Streptomyces* sp. P20. The strategy adopted to modify the usual literature media was: (1) fractional factorial design (FFD) to elucidate the key medium ingredients (2) central composite design (CCD) to optimise the concentration of the key components. Optimisation of the medium resulted in not only an 86% increase in microbial transglutaminase activity as compared to the media cited in the literature, but also a reduction in the production cost. Optimal fermentation conditions - namely temperature and agitation rate - were also studied, using CCD methodology. Usual conditions of 30°C and 100 rpm were within the optimal area. All other parameters for enzyme production were experimentally proven to be optimum fermentation conditions.

Keywords: *Streptomyces* sp. P20, medium optimization, microbial transglutaminase, fractional factorial design, central composite design, temperature and rotation optimization.

INTRODUCTION

Transglutaminase (EC 2.3.2.13; protein-glutamine γ-glutaminyltransferase) is an enzyme that catalyses an acyl transfer reaction using protein or peptide-bond glutamine residues as acyl donors and several primary amines as receptors. When the ε-amino groups of the protein or peptide-bond lysyl residues are present as acyl receptors, this enzyme is capable of forming intra and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (Soares et al., 2003).

The covalent cross-links between a number of proteins and peptides introduced by transglutaminase promote modification of the functional properties of the food proteins (Yokoyama et al., 2004). Therefore, transglutaminase are widely used by food-processing industries for the purpose of, new product development, modification of the product properties such as viscosity, emulsification foaming and nutritional values (Zhu et al., 1995 and Kwan and Easa, 2003).

Transglutaminases are found in mammalian tissues, plasma, fish, and plants (Pasternack et al., 1998). The mammalian enzymes are Ca^{+2}-dependent. However, the relatively small quantities and the complex separation and purification procedures for these enzymes led to the search for alternative microbial sources. The first microbial transglutaminase (MTGase) was obtained from actinomycetes (Ando et al., 1989). Since then, efforts have been made to obtain this enzyme in large quantity for commercial applications, especially for the enzymes from *Streptomyces* (Gerber et al., 1994; Zhu et al., 1998 a and b; Zotzel et al., 2003b; Yan et al., 2005).

So far, researches have been focused on the isolation and screening of microorganisms with transglutaminase activity, and the purification and characterization

of the enzymes. The media compositions used to produce MTGase from *Streptomyces* have been almost the same in all the papers published since Ando et al., 1989 (Meiying et al., 2001; Meiying et al., 2002; Souza et al., 2006). The formulation of the culture media is of critical importance for industrial processes because the composition affects product concentration, yield and volumetric productivity. It is also important to reduce the cost of the medium, as this may affect the overall process economics (Liu et al., 2005).

The traditional one-at-a-time optimization strategy is simple and useful for screening, and the individual effects of medium components can be seen without the use of sophisticated statistical analyses. Unfortunately, this simple method frequently fails to locate the region of optimum response because the joint effects of factors on the response are not taken into account. It has been reported that the complexities and uncertainties associated with large-scale fermentation usually come from a lack of knowledge of the sophisticated interactions among various factors affecting fermentation (Junqua et al., 1997).

Statistically based experimental designs provide an efficient approach for the optimization of media composition. The fractional factorial design (FFD) is especially suitable to account for the interactions and identify the most significant components in the medium formula. A combination of factors generating a certain optimal response can be identified via a factorial design and the use of response surface methodology (Lim et al., 2008).

Response surface methodology (RSM) is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the studies using one variable at a time. Also, significant interactions between variables can

be identified and quantified by this technique (Zuo and Lee, 2006; Yu et al., 2005; Kuster and Williams, 1964).

Taking into account that the soil is a great reservoir of actinomycetes and that there are few reports concerning the optimization of culture media for transglutaminase production, in this paper we report the isolation and screening of Brazilian soil actinomycetes for transglutaminase activity, and the fermentation conditions in order to maximize the transglutaminase yield.

MATERIALS AND METHODS

Materials

Yeast extract, malt extract, peptone and agar were purchased from Difco (Detroit, Mich., U.S.A). KH_2PO_4, $MgSO_4.7H_2O$, glycerol, glucose, were from Merck (Darmstad, Germany) N-carbo-benzoxy-L-glycine and L-glutamic acid γ-monohydroxamate were purchased from Sigma (St. Louis, Mo., U.S.A.). Soybean flour and potato starch were from Yasmin food industry (São Paulo, SP, Brazil) and Yoki food industry (São Bernardo do Campo, SP, Brazil), respectively.

Selective isolation and preservation of actinomycetes

The actinomycetes strains used in this study were isolated from soil samples collected in the States of São Paulo (Campinas and metropolitan area) and Bahia (Praia do Forte) in Brazil.

About five grams of the soil sample were added to 10 mL sterilized distilled water and the suspension shaken at 200 rpm for 10 min. Aliquots were inoculated onto

solidified starch-casein media (Folk and Cole, 1966). The plates were incubated at 30ºC for up to 5 days. Actinomycete-like colonies were streaked onto slants of ISP2 media (0.4% yeast extract, 1% malt extract, 0.4% de glucose and agar, pH 7.0 ± 0.2) and single colonies were obtained. Colonies were removed from the agar media and preserved in cryotubes with 10% glycerol solution at -80ºC.

Microorganisms selection for MTGase production

The ability to produce MTGase was determined by inoculating 1 mL of spore suspension into 250 mL Erlenmeyer flasks containing 50 mL of seed medium (Ando et al, 1989) composed of: 0.2 % peptone; 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 0.5% glucose (pH 7.0). The flasks were incubated for 2 days at 30°C and 200 rpm in a rotational shaker. Aliquots of 15 mL of pre inoculum were transferred to 135 mL of the basal medium (Ando et al, 1989) (2 % peptone; 0.2% yeast extract; 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% potato starch; 0.5% glucose; pH 7.0) in 500 mL Erlenmeyer flasks and cultivated at 30°C for 5 days at 200 rpm. All runs were made in duplicates.

Aliquots of 1 mL of culture medium were taken, and after centrifugation the enzyme activity in the supernatant was detected by the colorimetric hydroxamate procedure with N-carbo-benzoxy-L-glutaminyl-glycine according to Folk and Cole, 1966, with some modifications. One unit of MTGase activity was defined as the amount of enzyme causing the formation of one micromole of hydroxamic acid per min at 37°C. A calibration curve was prepared using L-glutamic acid γ-monohydroxamate.

Preliminary tests (Ver Anexo I para testes complementares)

Production of MTGase using a high viscosity medium

The production of MTGase was tested in a high viscosity medium composed of: 2% peptone; 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% potato starch; 0.2% glucose; and 2% soybean flour (pH 7.0). The contents of one cryotube (two agar mycelium cylinders of nearly 6mm in diameter) were transferred to 50 mL Erlenmeyer flasks containing 15 mL of medium and incubated for 5 days at 100 rpm and 30°C in a rotatory shaker. A 1 mL aliquot was taken after 120 hr, and the enzyme activity measured as described above.

Selection of the carbon and nitrogen sources for MTGase production

The effects of different nitrogen and carbon sources on MTGase production were investigated with the one-by-one time strategy. The nitrogen sources and corresponding concentrations tested were: 2% peptone with 0.2% yeast extract; 2% peptone; 2% corn steep liquor (CSL); and 2% casein. The carbon sources were: 2% potato starch with 0.2% glucose; 2% molasses; 2% sucrose; 2% maltodextrin; 2% glycerol; and 2% soluble starch. In the investigation of the nitrogen sources, growth was carried out in the medium containing: 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% soybean flour; 2% potato starch and 0.2% glucose. In the process of screening the carbon sources, fermentation was carried out using the medium containing: 0.2% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 2% soybean flour and 2% peptone.

The optimization procedure for the production parameters and experimental design

All experimental design results were analyzed using STATISTICA 5.0 for Windows (Statsoft, Inc.).

Elucidation of the significant components using a fractional factorial design (FFD)

A $2^{(6-2)}$ fractional factorial design was employed to determine the key ingredients that significantly affected MTGase production. There were six nutrient factors in the medium: peptone, KH_2PO_4, $MgSO_4.7H_2O$, potato starch, soybean flour and glucose. Each factor was examined at a high level (coded +1) and a low level (coded -1), which corresponded to the basal level ±50%, respectively. The center points were the trials under the basal level conditions (coded 0). Table 2.1 shows the variables and levels in detail. A ¼ fraction of the full factorial design was adopted and consequently the experiment included 16 (2^{6-2}) combinations plus two replicates at the center point, as shown in Table 2.1. The enzyme activity was measured after 72 hr, 96 hr and 120 hr of fermentation.

Optimization of key ingredient concentrations using a central composite design (CCD)

The medium components that significantly affected MTGase production were optimized using a CCD design. The variables were coded according to the equation (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the coded variable of a nutrient factor, X_i is the natural variable of the nutrient factor, X_o is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels are shown in Table 2.2.

The statistical software defined a full CCD design for 4 factors consisting of 24 combinations plus the replicates at the center point (16 cubic points, 8 star points and 2 replicates at the center point to estimate the experimental error and to investigate the suitability of the proposed model), and the details are given in Table 2.2. Once the

experiments had been performed, the experimental results were fitted to a second-order polynomial function. The Student t-test permitted the statistical significance of the regression coefficients to be checked, and the analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The model for the response was expressed in terms of coded variables, without taking the statistically non-significant terms into account.

Temperature and agitation optimization procedure and experimental design

The fermentation parameters that influenced the MTGase production were optimized using a CCD design. The variables were coded, as described previously, according to equation (1), and the variables and their levels are shown in Table 2.5.

The Statistica software defined a full CCD design for 2 factors (2^2), consisting of 9 combinations plus the replicates at the center point (5 cubic points, 4 star points and 3 replicates at the center point, to estimate the experimental error and investigate the suitability of the proposed model), and the details are presented in Table 2.5. The experimental results were fitted to a second-order polynomial function and the student t-test permitted checking of the statistical significance of the regression coefficients. The analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms.

Influence of inoculum size

The influence of inoculum size on MTGase activity was tested with a simple one-variable test. Five flasks of the optimum medium were inoculated with, respectively, one, two, three, four, and five cylinders (6mm Ø) of the fully-grown agar cultivation of

Streptomyces sp. P20 cultivated as described previously. All five tests were incubated in a rotatory shaker for 5 days under the best conditions of temperature and agitation, and the MTGase activity then measured. This procedure was carried out in triplicate.

Kinetic study

MTGase production by *Streptomyces* sp. P20 was observed for ten days. The microorganism was inoculated into ten 50 mL conical flasks containing 15 mL of optimized medium (2.5% peptone; 0.2% KH_2PO_4 ; 0.1% $MgSO_4.7H_2O$; 2% potato starch; 0.1% glucose; and 2% soybean flour).

RESULTS AND DISCUSSION

Microorganisms selection for MTGase production

Nearly 200 pure actinomycete cultures were investigated for MTGase production and the actinomycete strains T10b and P20 produced 0.15 $U.mL^{-1}$ and 0.25 $U.mL^{-1}$ of MTGase activity, respectively. The P20 strain was chosen as a potential producer of extracellular MTGase. The enzyme is calcium independent, which makes it much more interesting and suitable for industrial use (Yokoyama et al., 2004). The P20 strain was taxonomically identified by molecular methods (16S rDNA gene sequencing and phylogenetic analysis) as *Streptomyces* sp. and subsequently deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the number CBMAI 837, to be available for further investigations.

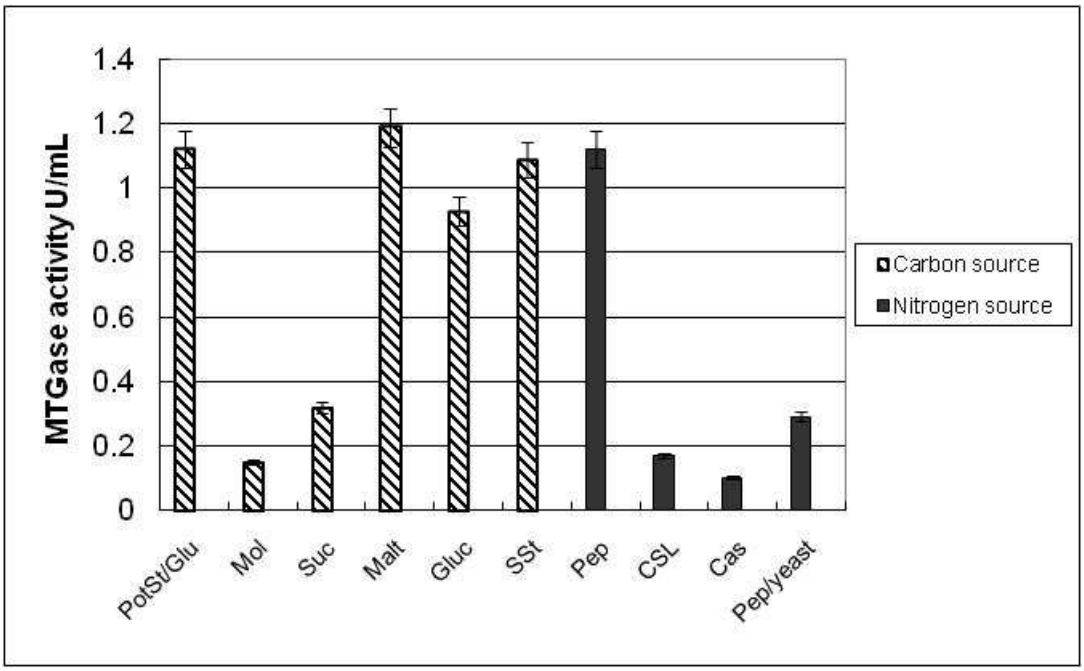
Preliminary tests

Production of MTGase using a high viscosity medium

In order to optimize the MTGase production, modifications of the usual media components reported for enzyme production were tested. *Streptomyces* sp. (strain P20) showed higher activity in the high viscosity medium (1.1 $U.mL^{-1}$) than in the basal medium (0.20 $U.mL^{-1}$) after 120 hr of fermentation. MTGase was more reliably produced in the high viscosity medium. These results further supported the hypothesis raised by Yan *et al*., 2005, that MTGase was probably involved in the formation of covalent bonds between different cell wall proteins to provide the mycelium of the *Streptomyces*.

Selection of the carbon and nitrogen sources for MTGase production

The effects of the carbon and nitrogen sources on the MTGase activity produced by *Streptomyces* sp. P20 are shown in Fig. 2.1. The data from these experiments showed that better results were obtained when peptone was used as the nitrogen source (1.12 $U.mL^{-1}$), and when a mixture of potato starch and glucose (1.12 $U.mL^{-1}$) or maltodextrin (1.18 $U.mL^{-1}$) was used as the carbon source. Both carbon sources were not significantly different and mixture of potato starch and glucose was chosen for further studies because of lower price.



PotSt/Glu: Potato starch/glucose; Mol: molasses; Suc: sucrose; Malt: maltodextrin; Gluc: glucose; SSt: soluble starch; Pep: peptone; CSL: corn steep liquor; Cas: casein; Pep/yeast: peptone/ yeast extract.

Figure 2.1. Effects of carbon and nitrogen sources on MTGase activity

Elucidation of the significant components using a fractional factorial design (FFD)

The next step was to define the media components that significantly affected the MTGase production and to determine the best concentrations of each. The most important nutrient factors were screened by applying the two-level fractional factorial design described in the material and methods section, and the experimental design and the results of the FFD observations are presented in Table 2.1. MTGase production varied greatly from 0.02 to 1.27 $U.mL^{-1}$ with different combinations of the media components, and the main effects are shown in Table 2.3. Observing the results of the FFD experiment, it was clear that variations in the concentrations of potato starch, peptone and glucose did not affect MTGase activity significantly at the levels tested. Thus the glucose concentration was fixed at the minimum value for the next factorial design, and the concentration of potato starch was fixed at an intermediary level. Since

peptone is the most expensive compound in the fermentation medium, its concentration was not fixed, but studied at a lower level of concentration in the next study. On the other hand, the enzyme production was greatly affected by soybean flour, KH_2PO_4 and $MgSO_4.7H_2O$ ($p<0.05$). These three nutrient components and peptone were further investigated in a broader range of concentrations within a CCD (Table 2.2).

Table 2.1. Coded levels and real values (in parentheses) for the experimental design and results of the 2^{6-2} fractional factorial design

Trial	*Soybean flour (%)*	*Potato starch (%)*	*Peptone (%)*	*KH_2PO_4 (%)*	*$MgSO_4.7H_2O$ (%)*	*Glucose (%)*	*MTGase activity (U/mL)*		
	x_1 (X_1)[1)]	x_2 (X_2)	x_3 (X_3)	x_4 (X_4)	x_5 (X_5)	x_6 (X_6)	72h	96h	120h
1	-1 (0,5)	-1 (0,5)	-1 (1,0)	-1 (0,0)	-1 (0,0)	-1 (0,1)	0,21	0,10	0,08
2	1 (2,5)	-1 (0,5)	-1 (1,0)	-1 (0,0)	1 (0,2)	-1 (0,1)	0,28	0,41	0,18
3	-1 (0,5)	1 (3,5)	-1 (1,0)	-1 (0,0)	1 (0,2)	1 (0,9)	0,02	0,51	1,08
4	1 (2,5)	1 (3,5)	-1 (1,0)	-1 (0,0)	-1 (0,0)	1 (0,9)	0,02	0,03	0,02
5	-1 (0,5)	-1 (0,5)	1 (2,0)	-1 (0,0)	1 (0,2)	1 (0,9)	0,02	0,32	0,22
6	1 (2,5)	-1 (0,5)	1 (2,0)	-1 (0,0)	-1 (0,0)	1 (0,9)	0,64	0,84	0,32
7	-1 (0,5)	1 (3,5)	1 (2,0)	-1 (0,0)	-1 (0,0)	-1 (0,1)	0,47	0,06	0,14
8	1 (2,5)	1 (3,5)	1 (2,0)	-1 (0,0)	1 (0,2)	-1 (0,1)	0,12	0,70	0,70
9	-1 (0,5)	-1 (0,5)	-1 (1,0)	1 (0,4)	-1 (0,0)	1 (0,9)	0,34	0,17	0,10
10	1 (2,5)	-1 (0,5)	-1 (1,0)	1 (0,4)	1 (0,2)	1 (0,9)	0,50	0,61	1,19
11	-1 (0,5)	1 (3,5)	-1 (1,0)	1 (0,4)	1 (0,2)	-1 (0,1)	0,63	1,15	1,14
12	1 (2,5)	1 (3,5)	-1 (1,0)	1 (0,4)	-1 (0,0)	-1 (0,1)	0,62	1,11	1,27
13	-1 (0,5)	-1 (0,5)	1 (2,0)	1 (0,4)	1 (0,2)	-1 (0,1)	0,03	0,05	0,04
14	1 (2,5)	-1 (0,5)	1 (2,0)	1 (0,4)	-1 (0,0)	-1 (0,1)	0,29	0,46	0,52
15	-1 (0,5)	1 (3,5)	1 (2,0)	1 (0,4)	-1 (0,0)	1 (0,9)	0,37	0,30	0,63
16	1 (2,5)	1 (3,5)	1 (2,0)	1 (0,4)	1 (0,2)	1 (0,9)	0,38	0,75	0,71
17	0 (1,5)	0 (2,0)	0 (1,5)	0 (0,2)	0 (0,1)	0 (0,5)	0,41	0,63	1,11
18	0 (1,5)	0 (2,0)	0 (1,5)	0 (0,2)	0 (0,1)	0 (0,5)	0,37	0,74	1,13
19	0 (1,5)	0 (2,0)	0 (1,5)	0 (0,2)	0 (0,1)	0 (0,5)	0,35	0,67	1,08

[1)] x_i is the coded value and X_i is the actual value of the *i*th independent variable. The conversion between x_i and X_i is described on equation (1).

Table 2.2 Coded levels and real values (in parentheses) for the experimental design and results of CCD for media compounds

Trial	*Soybean flour (%) $x_1(X_1)$*[1)]	*KH_2PO_4 (%) $x_2(X_2)$*	*$MgSO_4$ (%) $x_3(X_3)$*	*Peptone (%) $x_4(X_4)$*	*MTGase (U/mL)*	*120hr*
					Experimental	Predicted
1	-1 (1,5)	-1 (0,2)	-1 (0,1)	-1 (0,5)	0,87	0,78
2	1 (3,5)	-1 (0,2)	-1 (0,1)	-1 (0,5)	0,74	0,71
3	-1 (1,5)	1(0,6)	-1 (0,1)	-1 (0,5)	0,51	0,57
4	1 (3,5)	1(0,6)	-1 (0,1)	-1 (0,5)	0,99	0,93
5	-1 (1,5)	-1 (0,2)	1 (0,3)	-1 (0,5)	0,67	0,86
6	1 (3,5)	-1 (0,2)	1 (0,3)	-1 (0,5)	0,72	0,79
7	-1 (1,5)	1(0,6)	1 (0,3)	-1 (0,5)	0,81	0,64
8	1 (3,5)	1(0,6)	1 (0,3)	-1 (0,5)	1,01	1,00
9	-1 (1,5)	-1 (0,2)	-1 (0,1)	1 (1,5)	1,33	1,05
10	1 (3,5)	-1 (0,2)	-1 (0,1)	1 (1,5)	0,70	0,69
11	-1 (1,5)	1(0,6)	-1 (0,1)	1 (1,5)	0,82	0,83
12	1 (3,5)	1(0,6)	-1 (0,1)	1 (1,5)	0,78	0,90
13	-1 (1,5)	-1 (0,2)	1 (0,3)	1 (1,5)	0,36	0,50
14	1 (3,5)	-1 (0,2)	1 (0,3)	1 (1,5)	0,23	0,14
15	-1 (1,5)	1(0,6)	1 (0,3)	1 (1,5)	0,21	0,29
16	1 (3,5)	1(0,6)	1 (0,3)	1 (1,5)	0,44	0,36
17	-2 (0,5)	0 (0,4)	0 (0,2)	0 (1,0)	0,56	0,54
18	2 (4,5)	0 (0,4)	0 (0,2)	0 (1,0)	0,49	0,54
19	0 (2,5)	-2 (0,0)	0 (0,2)	0 (1,0)	0,57	0,71
20	0 (2,5)	2 (0,8)	0 (0,2)	0 (1,0)	0,81	0,71
21	0 (2,5)	0 (0,4)	-2 (0,0)	0 (1,0)	0,90	1,02
22	0 (2,5)	0 (0,4)	2 (0,4)	0 (1,0)	0,65	0,56
23	0 (2,5)	0 (0,4)	0 (0,2)	-2 (0,0)	0,91	0,91
24	0 (2,5)	0 (0,4)	0 (0,2)	2 (2,0)	0,49	0,53
25	0 (2,5)	0 (0,4)	0 (0,2)	0 (1,0)	1,43	1,37
26	0 (2,5)	0 (0,4)	0 (0,2)	0 (1,0)	1,17	1,37
27	0 (2,5)	0 (0,4)	0 (0,2)	0 (1,0)	1,50	1,37

[1)] x_i is the coded value and X_i is the actual value of the *i*th independent variable. The conversion between x_i and X_i is described on equation (1).

Table 2.3 Effects estimates, standard error, Student's t-test and p-test calculated for the 2^{6-2} fractional factorial design

	MTGase activity (U/mL)			
	Effec	Std. Err.	t-value	*p*-value
Mean/Inter.	0,3195[1)]	0,0061	52,5191	0,0004
Soybean flour (%)	0,0943[1)]	0,0133	7,1101	0,0192
Potato starch (%)	0,0384	0,0133	2,8980	0,1013
Peptone (%)	-0,0388	0,0133	-2,9244	0,0997
KH_2PO_4 (%)	0,1706[1)]	0,0133	12,8675	0,0060
$MgSO_4.7H_2O$ (%)	-0,1207[1)]	0,0133	-9,1024	0,0118
Glucose (%)	-0,0446	0,0133	-3,3609	0,0783

[1)]significant factors (p<0,05)

Optimization of key ingredient concentrations using a central composite design (CCD)

In order to optimize the key ingredients selected in the media, a CCD was carried out, and the experimental design and results are shown in Table 2.2. The quadratic model calculated for maximum MTGase activity, after eliminating the statistically insignificant terms (p>0.05), was:

$Y=1.3675-0.20602x_1^2-0.1652x_2^2-0.11622x_3-0.14376x_3^2-0.09508x_4-$

$.16281x_4^2+0.106293x_1.x_2-0.07399x_1.x_4-0.1557x_3.x_4$

The analysis of variance (ANOVA) reproduced in Table 2.4 showed that the model was significant. The Fisher F-test ($F_{9;17} = 14.2 > F_{t\ 0.95;9;17} = 2.49$) was 5-6 times higher than the Ft, and the p-value <0.000001 did, in fact, demonstrate that this regression was statistically significant at the 95% confidence level. In addition, the R^2 (multiple correlation coefficient) of the regression equation obtained was 0.88 (a value

>0.75 indicates aptness of the model), which means that the model can explain 88% of the variation in the response.

Table 2.4. Analysis of variance and regression analyses[1)] for the response of the 2^4 central composite design of the media compounds

Source of variation	*Sum of squares*	*Degrees of freedom*	*Mean squares*	F_{test}[2)]	*p-value*
Regression	2.4883	9	0.2765	14.2	< 0.00001
Residual	0.3310	17	0.0195		
Lack of fit	0.2733	15	0.0182		
Pure error	0.0577	2	0.0288		
Total	2.8193				

[1)]Coefficient of determination: R^2=0.88

[2)] $F_{0,95;9;17}$= 2.49

Three response surfaces were chosen amongst the possible combinations as representative of each selected rotation speed to visualize the simultaneous effects of peptone, soybean flour, KH_2PO_4 and $MgSO_4.7H_2O$ on the MTGase production pattern (Fig. 2.2).

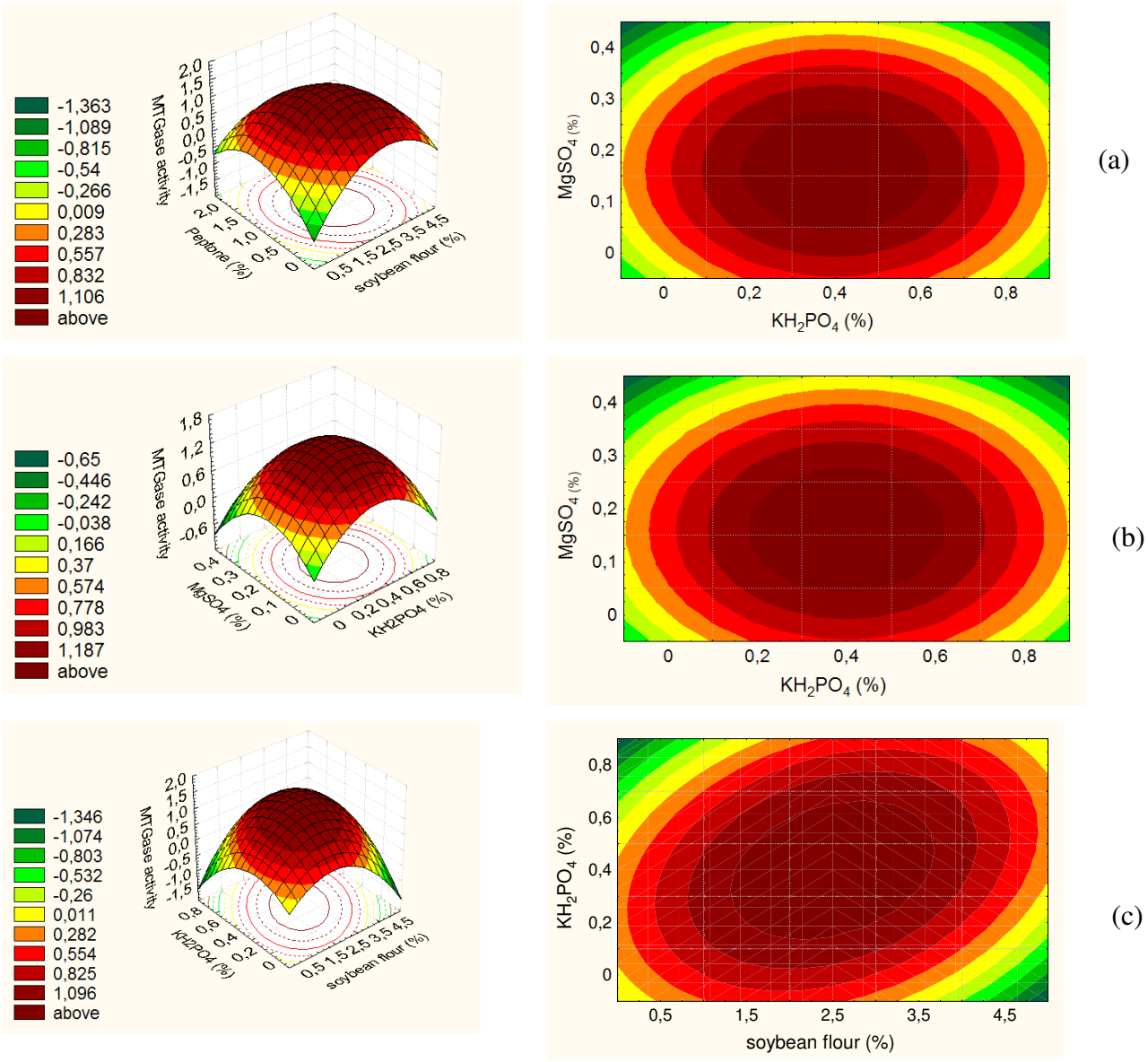


Figure 2.2. Contour curve and response surface for the MTGase activity as a function of: (a) peptone *versus* soybean flour (b) $MgSO_4$ *versus* KH_2PO_4 (c) KH_2PO_4 *versus* soybean flour concentrations, according to the CCD.

The results from the CCD showed that the optimal concentrations of the four key ingredients were basically the same as the values at the central point: 2.5% soybean flour; 0.4% KH_2PO_4; 0.2% $MgSO_4.7H_2O$ and 1.0% peptone. However, the reduction in peptone concentration decreased the MTGase activity. The maximum MTGase activity predicted by the model was calculated to be 1.37 $U.mL^{-1}$. In order to confirm the predicted results and validate the regression obtained, experiments using the improved formula of the medium were performed, and a value of 1.4 $U.mL^{-1}$ (n=3) was obtained, a result that demonstrated the adequacy of the model generated.

In the present work the fermentation medium for MTGase production was modified and optimized using statistical methods. The statistical tools, FFD and CCD, are found to be useful to determine the significant variables and the optimum conditions for MTGase production. The optimization of the medium resulted in not only an increase in MTGase activity from 0.25 $U.mL^{-1}$ to 1.4 $U.ml^{-1}$, but also a reduction in the costs of the constituents and an improvement in repeatability. It is important to note that the greatest relevance of these results lies in the factors leading to the increased activity, which may be important in further studies.

Temperature and agitation optimization procedure and experimental design

The study to determine the best conditions of temperature and agitation in the rotatory shaker was made using an experimental design and the results are shown in table 2.5. The quadratic model calculated for maximum MTGase activity, after eliminating the statistically insignificant terms (p>0.05), was:

$$Y=0.524018-0.13339x_1-0.177777x_1^2-0.30743x_2+0.114982x_1.x_2$$

Table 2.5. Coded levels and real values (in parentheses) for the experimental design and results of CCD for agitation and temperature

Trial	*Agitation (rpm)* $x_1(X_1)^{1)}$	*Temperature (°C)* $x_2(X_2)$	*MTGase (U/mL) 120hr*	
			Experimental	Predicted
1	-1 (115)	-1 (26)	1,5	1,26
2	1 (185)	-1 (26)	1,11	0,76
3	-1 (115)	1(34)	0,35	0,41
4	1 (185)	1(34)	0,42	0,37
5	-1,41 (100)	0 (30)	1,05	1,06
6	1,41 (200)	0 (30)	0,52	0,69
7	0 (150)	-1,41(25)	0,75	0,96
8	0 (150)	1,41(35)	0,32	0,09
9	0 (150)	0 (30)	0,51	0,52
10	0 (150)	0 (30)	0,54	0,52
11	0 (150)	0(30)	0,46	0,52
12	0 (150)	0(30)	0,59	0,52

1) x_i is the coded value and X_i is the actual value of the *i*th independent variable. The conversion between x_i and X_i is described on equation (1).

The analysis of variance reproduced in Table 2.6 shows that the model was significant. The Fisher *F*-test ($F_{4.7} = 6.67 > F_{t\ 0.95;4;7} = 4.12$) was 1.6 times higher than the *F*t, and the *p*-value of <0.01 demonstrated that this regression was statistically significant at the 95% confidence level. In addition, the R^2 obtained was 0.79.

Table 2.6. Analysis of variance and regression analyses[1)] for the response of the 2^2 central composite design of agitation and temperature

Source of variation	*Sum of squares*	*Degrees of freedom*	*Mean squares*	F_{test} [2)]	*p-value*
Regression	1,1338	4	0,2834	6,6714	< 0,01
Residual	0,2974	7	0,0424		
Lack of fit	0,2884	4	0,0721		
Pure error	0,0089	3	0,0029		
Total	1,4312				

[1)]Coefficient of determination: R^2=0,79

[2)] $F_{0,95;4;7}$= 4,12

The response surface obtained is shown in Fig. 2.3. It indicates that the highest MTGase activity values were found at the lowest levels of agitation and temperature tested. The best activity obtained in the experimental design was 115 rpm and 26°C.

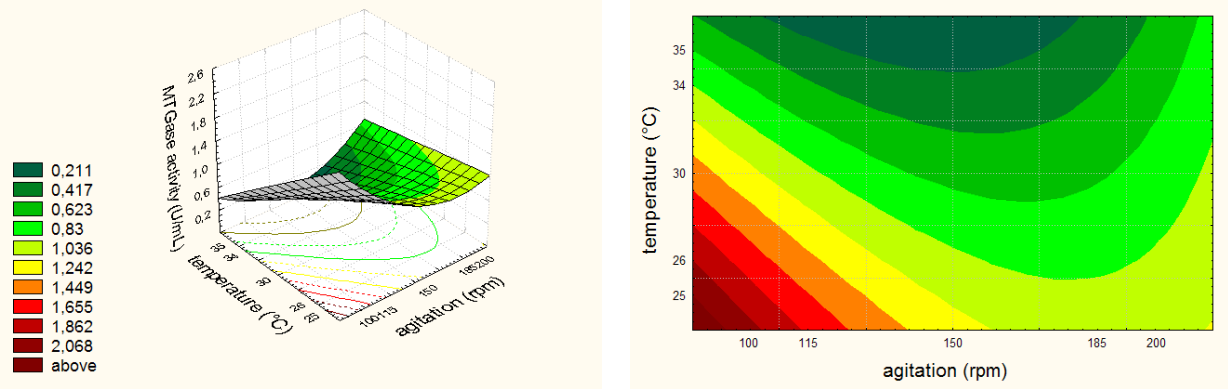


Figure 2.3. Contour curve and response surface for the MTGase activity as a function of: temperature *versus* agitation, according to the CCD.

The results from the CCD of the effects of temperature and rotation indicated higher MTGase production at 25-30°C or even lower temperatures. To confirm this, the Tukey test was applied to the means of the values obtained in the single tests under the following conditions: 100 rpm and 25°C, 100 rpm and 30°C and 100 rpm and 20°C. The

means were compared using the Tukey test at a confidence level of $p < 0.05$ and the tests were done in triplicate. The results indicated that at temperatures below 25°C, MTGase production was nearly zero. The other conclusion was that there was no significant difference in the MTGase activity obtained between 30 and 25°C. Thus the enzyme production conditions chosen were the value of 100 rpm indicated by the CCD and a temperature of 30°C.

Influence of inoculum size and kinetic study

The influence of inoculum size on MTGase production was tested and the results analysed using the Tukey test, admitting a 95% level of significance ($p \leq 0.05$), which indicated there was no statistical significant difference in the MTGase activity obtained using from one to five agar-mycelium cylinders as the inoculum. The data from the kinetic study of the fermentation process is described in Fig.2.4. The inoculum size and kinetic study results indicated that the pre-established parameters of a two agar-mycelium cylinder inoculum and five days of fermentation were completely adequate.

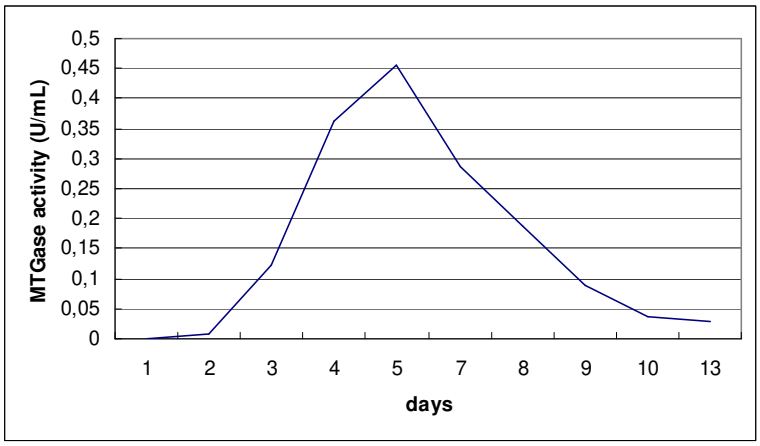


Figure 2.4. Production of MTGase during fermentation of *Streptomyces* sp. CBMAI 837

Considering the results, the Brazilian soil isolate *Streptomyces* sp. CBMAI 837 has great potential for further investigation with respect to MTGase production. Further experiments for the characterization and purification of the enzyme are being carried out.

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CAPÍTULO 3. PURIFICATION AND CHARACTERIZATION OF A NEW TRANSGLUTAMINASE FROM *STREPTOMYCES* SP. ISOLATED FROM BRAZILIAN SOIL

Juliana Alves Macedo[1*], Lara Durães Sette[2], Hélia Harumi Sato[1]

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[*] Corresponding author: Tel.: + 55 (19) 3521-2175; Fax +55 (19) 3289-1513.

[1] Food Science Department, Faculty of Food Engineering, UNIVERSIDADE ESTADUAL DE CAMPINAS (UNICAMP) P.O. Box 6121, CEP 13083-862, SP, Brazil.

[2] Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), Microbial Resource Division, CPQBA/UNICAMP, P.O. Box 6171, CEP 13081-970, SP, Brazil.

ABSTRACT

A new microbial transglutaminase (MTGase or MTG, EC 2.3.2.13) from a *Streptomyces sp*. strain isolated from Brazilian soil samples was purified and characterized. Enzyme purification was fast and simple, consisting of two successive chromatographies on Sephadex G-75 columns with yields of 48% and 17% respectively. The protein purification was successfully achieved to electrophoretical homogeneity on SDS-PAGE. The molecular mass of the MTGase was estimated to be about 45 kDa. The enzyme from *Streptomyces sp*., in both crude and pure forms, exhibited optimal activity in the 6.0-6.5 pH range and at 35-40°C. The results for the commercial enzyme were the same. A second maximum of activity was observed at pH 10.0 with both the crude *Streptomyces sp*. enzyme and the commercial enzyme. This interesting fact has not been reported in the literature previously. All of the enzymes tested were stable over the pH range from 4.5 to 8.0 and up to 45°C. The decline in activity of the commercial transglutaminase above 45°C and pH 8.0 was more gradual. The activities of all the MTGase samples were independent of Ca^{+2} concentration, but they were elevated in the presence of K^{+}, Ba^{2+}, and Co^{2+} and inhibited by Cu^{2+} and Hg^{2+}, which suggests the presence of a thiol group in the MTG's active site. The purified enzyme presented a K_m of 6.37 mM and a V_{max} of 1.7 U/mL, while the crude enzyme demonstrated a K_m of 6.52 mM and a V_{max} of 1.35 U/mL.

Keywords: transglutaminase, *Streptomyces* sp. CBMAI 837, enzyme purification, Sephadex G-75, biochemical characterization.

INTRODUÇÃO

Transglutaminase (EC 2.3.2.13; protein-glutamine γ-glutaminyltranferase) is an enzyme that catalyses an acyl transfer reaction using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. When the ε-amino groups of the protein-bond lysyl residues are present as acyl receptors, this enzyme is capable of forming intra-and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (Soares et al., 2003).

The covalent cross-links between a number of proteins and peptides introduced by transglutaminase promote modification of the functional properties of food proteins (Yokoyama et al., 2004). Therefore, transglutaminase-catalyzed reactions may be widely used by food-processing industries in the creation of new products, modification of the viscosity, alterations in the emulsifying and foaming properties, and improvement of the product‘s nutritional value (Zhu et al., 2004; Kwan & Easa, 1995).

Transglutaminases are found in mammalian tissues, plasma, fish, and plants (Pasternack et al., 1998). The mammalian enzymes are Ca^{+2}-dependent. However, the relatively small quantities obtained and the necessary complex separation and purification procedures led to the search for alternative microbiological sources such as *Candida albicans*, *Bacillus circulans*, *Physarum polycephalum* and many *Streptomyces* species (Yokoyama et al., 2004; Zhu et al., 1995; Herrera et al., 1995; Souza et al., 2006; Klein et al., 1992). Ando et al. (1989) first reported that strains from the genus *Streptoverticillium* screened from several thousand microorganisms had the ability to produce transglutaminase using the hydroxamate assay. These microorganisms excreted the enzyme, and one of them, which was classified as a variant of *Streptoverticillium*

mobaraensis, had high activity (Cui, Du, Zhang, Liu & Chen, 2007). The enzyme from microorganisms was named microbial transglutaminase (MTGase or MTG).

The MTGase from *Streptomyces* has been produced at an industrial scale by Ajinomoto Co., Inc., with the collaboration of Amano Enzyme, Inc.

The screening work and optimal conditions for the production of MTGases in a rotatory shaker have been described (Macedo, et.al., 2007; Macedo, et al., 2008). We recently isolated a new microbial strain from Brazilian soil samples, which is classified as *Streptomyces* sp. CBMAI 837. The objectives of this work were to purify and characterize the MTGase from the newly isolated *Streptomyces* sp. CBMAI 837. The scaled-up cultivation, downstream process and enzyme application are being investigated and are in the advanced stages of study (unpublished data).

MATERIAL AND METHODS

Material

Yeast extract, malt extract, peptone and agar were purchased from OXOID (Basingstone, Hants, England). KH_2PO_4, $MgSO_4.7H_2O$, glycerol, and glucose were obtained from Synth (Detroit, MI., U.S.A). N-carbo-benzoxy-L-glycine and L-glutamic acid γ-monohydroxamate were purchased from Sigma (St. Louis, MO, U.S.A.). Soybean flour and the potato starch were obtained from Yasmin Food Industry (São Paulo, SP, Brazil) and Yoki Food Industry (São Bernardo do Campo, SP, Brazil), respectively. The low molecular mass protein calibration kit and the Sephadex G-75 resin were from GE Healthcare. Commercial transglutaminase (Activa® TG-BP) was provided from Ajinomoto Co., Inc.

Microorganism and crude enzyme preparation (ver Anexo II para testes complementares)

Streptomyces sp. CBMAI 837, which was collected by our laboratory, was used throughout this study. The strain was cultivated in petri dishes containing ISP2 media (0.4% yeast extract, 1% malt extract, 0.4% glucose and 1.5% agar, pH 7.0 ± 0.2) for 7 days at 30°C, and two cylinders (6mm Ø) of the fully grown agar cultivation were removed from the petri dishes and preserved in cryotubes with a 10% glycerol solution at -80ºC. The content of one cryotube was inoculated into a 50 mL Erlenmeyer flask with 15 mL of fermentation medium and cultivated at 30°C for 5 days at 200 rpm in a rotatory shaker (Tecnal TE-421). The optimized fermentation medium consisted of 0.2% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, 2% soybean flour, 2% potato starch, 0.2% glucose, and 2% peptone (Macedo et al., 2007). After five days, the culture broth was centrifuged at 10,000 x *g* for 15 min at 5°C to remove microorganisms, and the resulting supernatant solution was frozen and lyophilized. The enzymatic preparation was stored at -20 ºC.

Analytical methods

Transglutaminase activity

The transglutaminase activity was determined by hydroxamate formation with the specific substrate N-carbobenzoxy-L-glutaminyl glycine (N-CBZ-Gln-Gly) according to Folk & Cole, 1966, with some modifications. The reaction mixture, containing 200 µL of enzyme, 200 µL of 0.2 M citrate buffer pH 6.0, 25 µL of hydroxylamine, and 75 µL of 0.1 M N-carbobenzoxy-L- glutaminyl glycine, was incubated at 37°C for 1h and then stopped by adding an equal volume (500 µL) of 15% TCA containing 5% $FeCl_3$. The absorbance was measured at 525 nm (Beckman Coulter DU 640). One unit of MTGase

activity was defined as the amount that causes the formation of one micromole of hydroxamic acid per minute at 37°C. A calibration curve was prepared using L-glutamic acid y-monohydroxamate.

Protein determination

The amount of protein was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Purification of MTGase (ver Anexo III para estudos complementares)

All the steps were performed at 6°C, unless otherwise stated.

Sephadex G-75 chromatography

A 1 g sample of crude enzyme powder (0.52 U of transglutaminase) was dissolved in 5 mL of 25 mM phosphate buffer, pH 6.0 and was applied on a Sephadex G-75 column (1.10 x 95 cm) pre-equilibrated with the same buffer. The column was washed with 0.1 M NaCl in the same buffer at a flow rate of 1.0 mL/15 min, and the active fractions were collected and pooled. Ten militers of the active pooled fraction obtained from the Sephadex G-75 column were applied to the same column under identical conditions, and the active fractions were pooled, lyophilized and stored at -20 ºC.

Sodium dodecylsulfate polyacrylamide gel electrophoresis

The purified enzyme was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Vertical Slab Mini-Protean Electrophoresis System Bio-Rad Laboratories, Hercules, CA, USA) according to Laemmli, (1970). A 12% separating gel was used. The proteins were stained with a 0.1% solution of Coomassie brilliant blue R-250.

Biochemical characterization of MTGase

All procedures were carried out under the same conditions for samples of both the commercial MTGase and the pure and crude MTGase from *Streptomyces sp*. CBMAI 837. In order to compare the results obtained for each enzyme sample, the amount of enzyme powder in each trial was defined as the total measured protein in the sample. The total amount of protein present in each reaction was held constant (4 μg protein). All experiments were run in triplicate.

pH effect

The effect of pH on the MTGase activity was determined under standard assay conditions using a 50 mM sodium citrate buffer (pH 3.0-6.5), a 50 mM Tris-HCl buffer (pH 7.0-9.0) and a 50 mM Borax-NaOH buffer (pH 10.0). The enzyme activity was measured after 60 min at 37°C.

The effect of pH on MTGase stability was determined using the above mentioned buffer systems over the pH range 3.0-10.0. The enzyme solutions were incubated at the various pH values for 60 min at 25ºC without substrate. The remaining enzyme activity was then measured at 37ºC using N-CBZ-Gln-Gly in a sodium citrate buffer at pH 6.0. The relative activities were determined by using the maximal activity of the enzyme at a specific pH as 100%.

Temperature effect

The effect of temperature on MTGase activity was tested by assaying the activity at different temperatures (4ºC and over the range from 25°C to 70°C at pH 6.0) using the reaction mixtures indicated previously. The relative activities were determined by defining the maximal activity of the enzyme at a specific temperature as 100%. The

enzyme's thermal stability was determined by pre-incubating it at various temperatures for 30 min, and then placing the samples on ice immediately. The relative activities were then determined using the standard method described above.

The effect of metal ions

The effects of different metal ions were investigated by adding them (5 mM) to the reaction mixture and incubating at 37ºC for 60 min under the standard assay conditions. The relative activities were determined by using the activity of the enzyme in the absence of additives as 100%.

Kinetic parameters

Kinetic parameters were determined using the experimental system described above with varying amounts of the model substrate, N-CBZ-Gln-Gly (0-30 mM). The Michaelis constants (K_m) and maximum velocities (V_{max}) were determined from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Purification of MTGase (ver anexo III para estudos complementares)

The enzyme was purified 5.0 fold with a yield of 17.7% after two steps of gel filtration chromatography.

The crude enzyme preparation was submitted to the initial purification step, the first chromatographic run on a Sephadex G-75 column (Fig.3.1), which resulted in a 2.3-fold purification, a yield of 48%, and a specific activity of 1.92 U/mg (Table 3.1). The usual first run on a CM-cellulose column described for MTGase purification in several cases (Soares et al., 2003; Yokoyama et al., 2004; Zhu et al., 1995; Klein et al., 1992; Ando et al., 1989; Cui et al., 2007; Ho, Leu, Hsieh & Jiang, 2000.) was avoided because

the MTGase-ion exchange resins strongly affected the enzyme activity (data not shown). A further purification step was achieved by subjecting the pooled enzyme fraction from the first column step to a second run through the same Sephadex G-75 column (Fig. 3.1), which resulted in a 5.0-fold purification with a yield of 17.7% and a specific activity of 4.18 U/mg protein as compared to the crude extract (Table 3.1). Though both the purification fold and yield were lower after this step, protein purification was successfully achieved to SDS-PAGE electrophoretical homogeneity. From these results, the molecular mass of the MTGase from *Streptoverticillium* sp. CBMAI 837 was estimated to be about 45 kDa (Fig. 3.2). This result shows that the enzyme is heavier than that from *Bacillus subtillis* (29 kDa) (Suzuki et al., 2000), *Streptomyces mobaraensis* (37 kDa) (Ando et al., 1989), and *Streptomyces hygroscopicus* (38 kDa) (Cui et al., 2007), and approximately equal to those from *Streptoverticillium* sp S 8112 (40 kDa) (Ando et al., 1989) and *Bacillus circulans* (45 kDa) (Soares et al., 2003). However, it is smaller than other Ca^{+2}-dependent tissue (Guinea pig liver, 90 kDa and tropical tilapia, 85 kDa) transglutaminase (Folk and Cole, 1966; Worratao & Yongsawatdigul, 2000).

This purification method proved to be rapid and practical, which are very important characteristics for downstream enzymatic methodology.

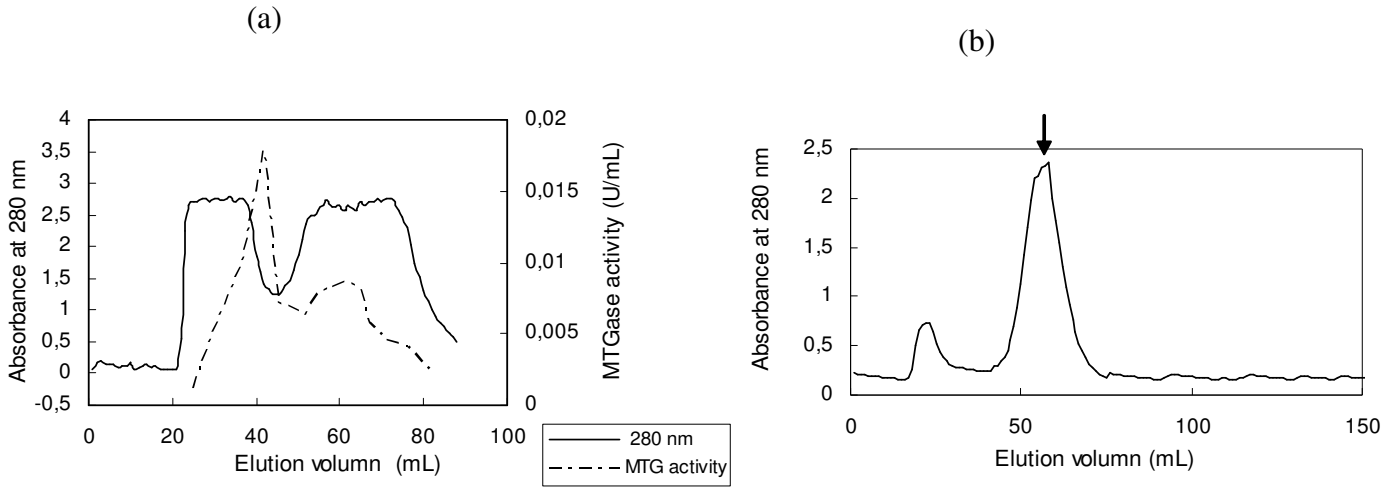


Figure 3.1. Chromatography of the crude MTG from *Streptomyces* sp. CBMAI 837 on: (a) the first and (b) second run on the Sephadex G-75 column. The arrow indicates the fraction containing MTG activity.

Table 3.1. Summary of purification of MTG from *Streptomyces* sp. CBMAI 837 on Sephadex G-75 column.

Purification step	***MTG (U)***	***Protein (mg)***	***Specific activity (U/mg)***	***Purification fold***	***Yield (%)***
Solution of lyophilized crude enzyme extract (1.0 g/5 mL)	0.520	0.625	0.83	1	100
Sephadex G-75 (first run)	0.250	0.130	1.92	2.3	48
Sephadex G-75 (second run, lyophilized MTG active fractions in solution, 150 mg/mL)	0.092	0.022	4.18	5.0	17.7

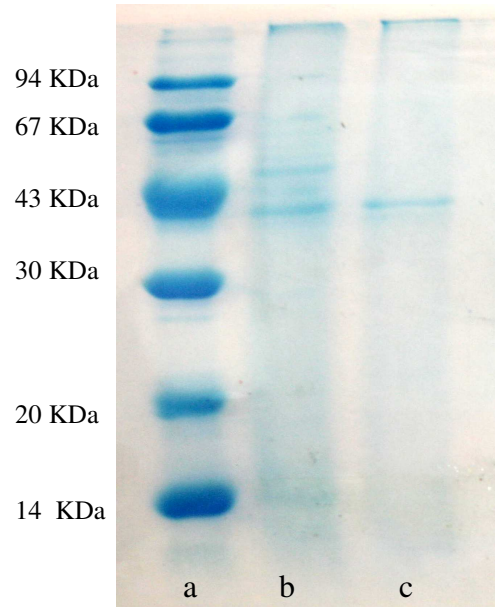


Figure 3.2. SDS-PAGE of MTG on an acrylamide gel showing the (a) standard marker, (b) crude enzyme, and (c) MTG after the Sephadex G-75 second run.

Effect of pH on MTGase activity

The effect of pH on the activity of the different MTG samples was determined using the previously described reaction mixtures in the pH range from 3.0 to 10.0 at 37°C. The three different samples exhibited optimum activity for the catalytic reaction with N-CBZ-Gln-Gly in the 6.0-6.5 pH range. But the crude *Streptomyces sp*. enzyme and the commercial MTGase sample demonstrated a second maximum of activity at pH 10.0 (Fig. 3.3 (a)). This test was repeated several times, and control samples used to test for the possible degradation of the substrate at this alkaline pH developed no color. There is the possibility that two different transglutaminases were present in the unpurified sample. More experiments are needed to determine the properties of the enzyme that is active at pH 10.0.

The pH stability tests revealed that the purifyed and the commercial MTGase samples were stable from pH 5.0 to 8.0 (Fig. 3.3 (b)), but the commercial enzyme suffered a more gradual loss of activity above pH 8.0 than the other samples. The crude MTGase sample lost 20% of its enzymatic activity from pH 6.0-8.0. The optimum pH for these enzymes, about 6.0, was nearly the same as that reported for *Streptomyces hygroscopicus* (Cui et al., 2007). However, there are no literature reports of a second maximum in activity at pH 10.0. The activity of all three enzyme samples decreased gradually at alkaline pH values, but it decreased rapidly with increasingly acidic pH values.

The corresponding enzymes from mammals and fish have an optimum pH value of 8.0, while soybean MTGase has been reported to have an optimal pH value of 7.6 (Zhu et al., 1995; Worratao & Yongsawatdigul, 2000).

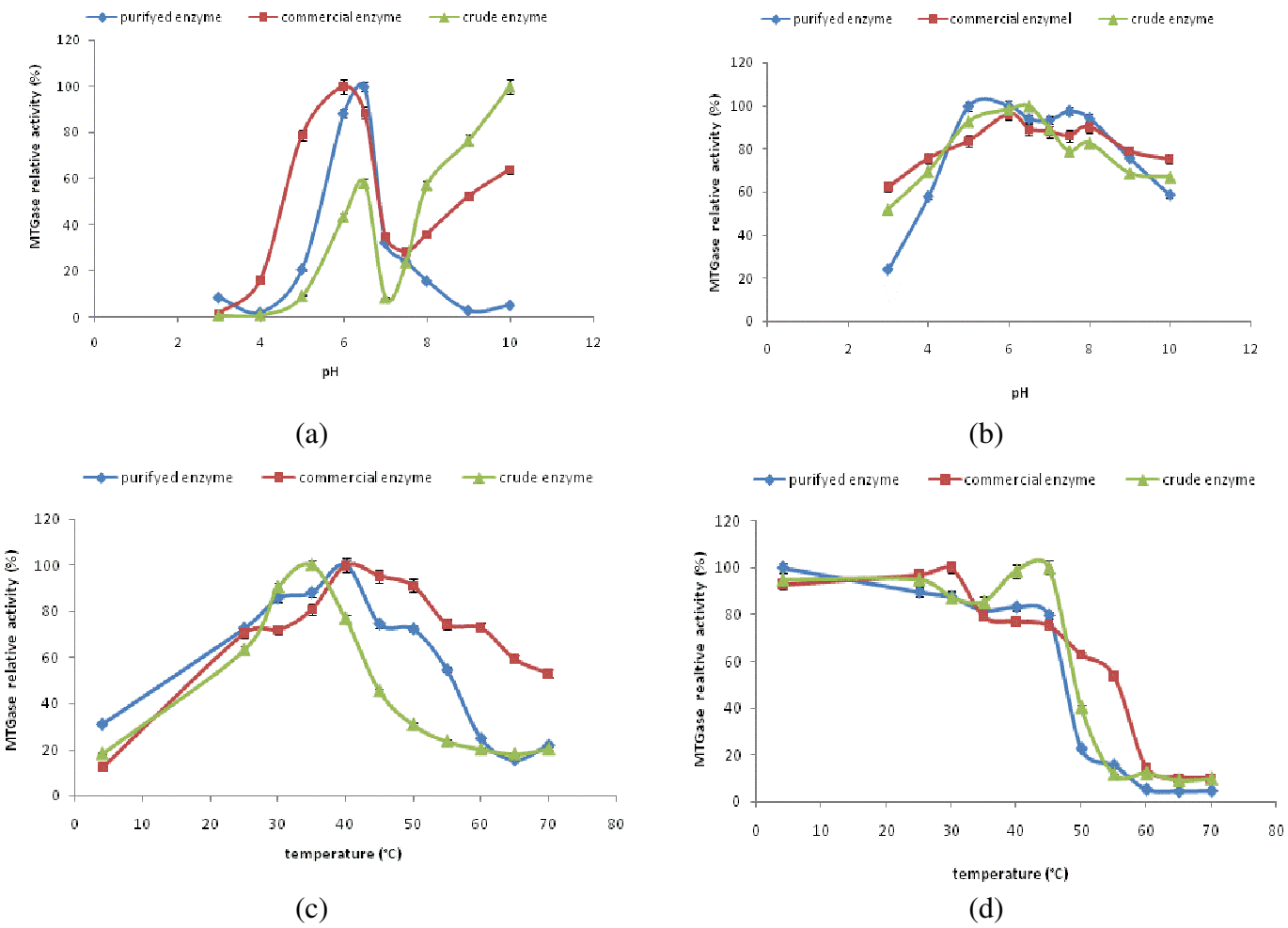


Figure 3.3. Effect of pH on the (a) activity and (b) stability and the effect of temperature on (c) activity and (d) stability of: purified and crude MTG from *Streptomyces* sp. CBMAI 837, and commercial MTG.

Effect of temperature on MTGase activity

The effects of temperatures on the activity of the MTG samples were studied by determining the activity of the samples at 4°C and at temperatures between 25-70°C at pH 6.0, under the conditions previously indicated. All the enzyme samples showed optimum activity in catalyzing the reaction of N-CBZ-Gln-Gly and hydroxylamine at 35-40°C. Almost no enzyme activity was detected at 60°C for either the crude or purified

MTG from *Streptomyces* sp., but the commercial sample still displayed some residual activity (Fig. 3.3 (c)). The temperatures required for the optimum activity of these MTG enzymes are very similar to the optimum temperatures reported for *Streptomyces hygroscopicus* and *Streptoverticillium ladakanum* (Cui et al., 2007; Ho et al., 2000). However, the MGT enzyme from *Streptomyces sp*. is completely different from *Bacillus subtilis* transglutaminases, which have optimal temperatures of 60°C (Suzuki et al., 2000).

The thermal stability of the enzymes was investigated between 4 and 70°C, and on average, they retained about 80% of their activity in the temperature range from 4 to 45°C after a 30 min incubation at pH 6.0. The activity of the purified enzyme was nearly zero and for the other two samples decreased to 10%, after incubation at 60°C (Fig.3.3 (d)).

Effect of different inhibitors and metal ions on MTGase activity

The relative activity of the transglutaminases was investigated in the presence of several metal ions and EDTA (ethylenediaminetetraacetic acid), which were added in different concentrations according to the method described above. As shown in Table 3.2, all three MTG samples were strongly inhibited by Cu^{2+} and Hg^{2+}. These metal ions are known to react preferentially with thiol groups, and the strong inhibition of the enzyme by these ions suggests that this enzyme contains a thiol group in its active site, similar to other TGases from both vertebrate tissues and microorganisms (Soares et al., 2003; Ando et al., 1989; Ho et al., 2000). On the other hand, the presence of K^{+}, Ba^{2+}, and Co^{2+} led to an increase in the activity of the crude and purified extracts from *Streptomyces sp*. MTG. However, these same metal ions decreased the MTG activity of the commercial enzyme.

These differences may be significant in some industrial processes, and this demonstrates the importance of characterizing new enzymes. The presence of Ca^{2+} increased the MTG activity in all of the samples, but only by 10-19%, which suggests that these enzymes are calcium independent.

Under the same incubation conditions and metal ion concentrations, the enzyme from *Streptomyces hygroscopicus* was strongly inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and Fe^{3+} (Cui et al., 2007).

The effect of EDTA on the activity of the MTG samples is shown in Table 3.3. The different MTG samples reacted very differently to the presence of EDTA. The commercial enzyme was unaffected by the presence of EDTA at all of the concentrations tested. The purified MTG extract from *Streptomyces sp*. showed decreased activity as the EDTA concentration increased. There was a 30% loss of enzyme activity when the EDTA concentration was increased from 0 to 5 mM. However, the crude MTG extract from *Streptomyces sp*. did not show a difference in activity until the EDTA concentration reached 5 mM, at which point the activity decreased by about 50%.

Table 3.2. The influence of various metal ions on the activity of purified and crude *Streptomyces* sp. CBMAI 837 transglutaminase and commercial transglutaminase.

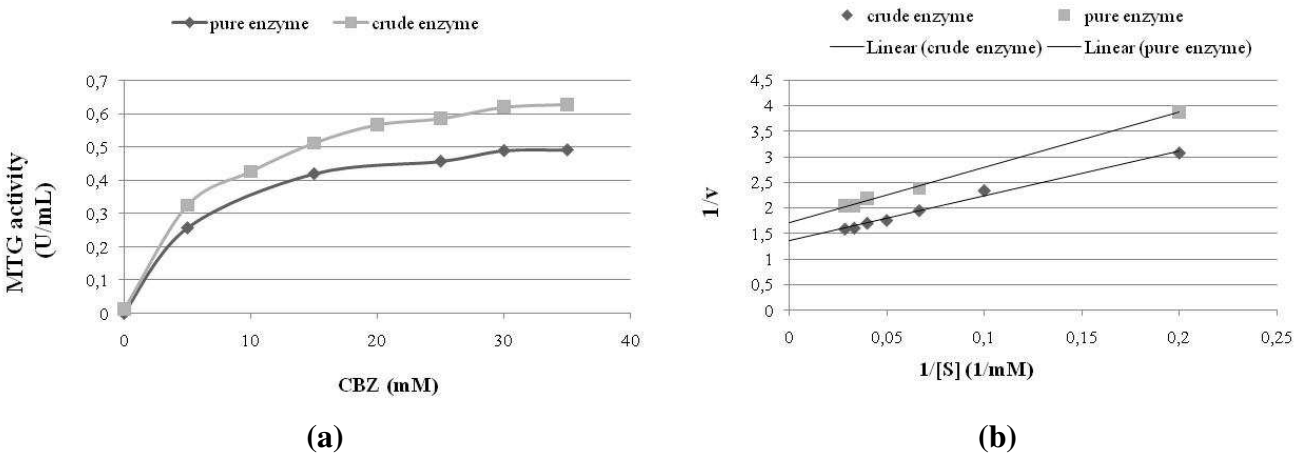
	MTG relative activity (%)		
Metal ion (5mM)	Crude enzyme	Pure enzyme	Commercial enzyme
Co^{2+}	132	118	85
Cu^{2+}	21	21	21
K^{+}	124	126	89
Ca^{2+}	118	112	119
Fe^{3+}	128	97	88
Zn^{2+}	118	74	138
Ba^{2+}	121	123	106
Hg^{2+}	9	11	11
Mg^{2+}	114	83	101
Na^{+}	108	118	107
Mn^{2+}	99	115	88
None	100	100	100

Table 3.3. The effect of EDTA on the activity of purified and crude *Streptomyces* sp. transglutaminase and commercial transglutaminase.

	MTG relative activity (%)		
EDTA (mM)	Crude enzyme	Pure enzyme	Commercial enzyme
0	100	100	100
0.1	111	105	108
0.5	98	83	105
1	100	96	97
5	53	70	103

Determination of kinetic parameters

The effect of substrate concentration on the velocity of the enzymatic reaction was determined at pH 6.0 and 37°C. The crude enzyme preparation from *Streptomyces sp.* presented a K_m of 6.52 mM and a V_{max} of 1.35 U/mL, while the purified enzyme presented a K_m of 6.37 mM and a V_{max} of 1.7 U/mL for the reaction with N-CBZ-Gln-Gly. These values were derived from the corresponding Lineweaver-Burk plots (Fig.3.4).



(a) **(b)**

Figure 3.4. Determination of kinetic parameters of the purified and crude MTG from *Streptomyces* sp. CBMAI 837 (a) Kinetic studies, (b) Lineweaver-Burk plot.

Under the same conditions, the transglutaminase from *Streptomyces hygroscopicus*, studied by Cui et. al., 2007, presented a K_m of 54.69 mM and a V_{max} of 1.28U/mL. And the *Streptomyces mobaraense* transglutaminase, studied by Gerber et al., 1994, showed a K_m of 12.2 mM. The lower values of K_m presented by both forms of the transglutaminase from *Streptomyces sp.* CBMAI 837, indicates a higher affinity of this enzyme for the substrate N-CBZ-Gln-Gly.

CONCLUSIONS

This paper reports the purification and characterization of transglutaminase from a newly isolated *Streptomyces* sp. CBMAI 837. The MTG was purified after two fast and simple steps on the same column, and the molecular mass of the enzyme was estimated to be 45 kDa. In terms of optimum activity and stability over a range of pH and temperature, the new transglutaminase performed in a manner very similar to the commercial transglutaminase. All of the samples exhibited optimal activity in the pH range 6.0-6.5 and at 35-40°C. A second maximum in activity was observed at pH 10.0 for both the crude *Streptomyces sp*. enzyme and the commercial enzyme. This interesting fact has not been previously reported in the literature. The crude preparation may contain two different transglutaminase.

All of the enzymes preparations tested were stable over a broad pH range (4.5-8.0) and up to 45°C. This biochemical characterization revealed that MTG from *Streptomyces* sp. CBMAI 837 had a pH response similar to the commercially available enzyme, which is good for food processing, because it is stable over a broad pH range with optimum activity near neutral pH. The catalytic activities of all the MTG samples were independent of Ca^{+2}, but they were enhanced in the presence of K^{+}, Ba^{2+}, and Co^{2+} and inhibited by Cu^{2+} and Hg^{2+}, which suggests a thiol group in their active sites. The purified enzyme demonstrated a K_m of 6.37 mM and a V_{max} of 1.7 U/mL, while the crude enzyme exhibited a K_m of 6.52 mM and a V_{max} of 1.35 U/mL.

The transglutaminase from *Streptomyces* sp. CBMAI 837 is a good candidate for applications in the food industry. However, additional work to increase the activity of the

enzyme and its yield during extraction and purification are required in order to commercialize the MTG from this newly isolated strain.

Acknowledgements

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CAPÍTULO 4. THE EFFECT OF TRANSGLUTAMINASE FROM STREPTOMYCES SP. CBMAI 837 ON GELATION OF ACIDIFIED SODIUM CASEINATE

Juliana Alves Macedo[*1], Angelo Luiz Fazani Cavallieri[2], Rosiane Lopes da Cunha[2], Hélia Harumi Sato[1]

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[*] Corresponding author: Tel.: + 55 (19) 3521-2175; Fax +55 (19) 3289-1513.

[1]Food Science Department, Faculty of Food Engineering, UNIVERSIDADE ESTADUAL DE CAMPINAS (UNICAMP) P.O. Box 6121, CEP 13083-862, SP, Brazil.

[2] Department of Food Engineering, Faculty of Food Engineering, UNIVERSIDADE ESTADUAL DE CAMPINAS (UNICAMP) P.O. Box 6121, CEP 13083-862, SP, Brazil.

ABSTRACT

The aim of this work was to study the influence of the transglutaminase enzyme from a newly isolated Brazilian *Streptomyces* sp. CBMAI 837 (Coleção Brasileira de Microrganismos de Ambiente e Indústria) and sodium caseinate concentration (4% and 8% (w/w)) on acid-gel properties. Standard gels with and without commercial transglutaminase (TG) samples were tested in parallel. The mechanical properties of the samples were evaluated using uniaxial compression measurements (stress and strain at rupture and elasticity modulus). Texture parameters showed that the commercial TG gels had greater values of elasticity modulus and could promote the formation of more elastic and soft food systems, while addition of the TG of *Streptomyces* sp. CBMAI 837 to the gel led to the formation of more rigid and less elastic gels. The electrophoresis results showed that the commercial TG enzyme in this system promoted higher molecular mass protein polymers than the enzyme from *Streptomyces* sp. CBMAI 837. Microscopy and water holding capacity (WHC) observations showed that all the gel characteristics could be associated to specific interactions promoted by each TG tested.

***Keywords*:** transglutaminase, *Streptomyces* sp. CBMAI 837, sodium caseinate, glucono-δ-lactone

INTRODUCTION

Texture is an important quality attribute of many milk products. Fermented milk products, such as yogurts, have a soft texture mainly due to the presence of physical cross-links in the protein matrix. Enzymes are potential tools to increase the formation of covalent cross-links in proteinaceous foods (Myllarine et al., 2007). Transglutaminase (TG; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme currently used in a variety of food structure engineering applications (Zhu et al., 1995). We have recently isolated a microbial strain from Brazilian soil samples classified as *Streptomyces* sp. CBMAI 837.

TG is an enzyme that catalyzes the transfer of an acyl group using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. When the ε-amino groups of lysyl residues are present as acyl receptors, this enzyme is capable of forming intra- and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (Soares et al., 2003). In the absence of amine substrates, transglutaminase is capable of catalyzing the deamidation of glutamine residues. In this reaction, water is used as nucleophile and ammonia is liberated.

To modify the techno-functional properties of food proteins, particularly with the aim of improving gel formation, water-binding, heat stability and rheological properties, enzymatic cross-linking by transglutaminase was carried out in a model system using substrates such as whey protein, casein, soy protein, fish protein as well as fractions of these proteins (Lorenzen et al., 1998). In contrast to some globular proteins, caseins are effective substrates for transglutaminase mainly because of their open tertiary structure. The sensitivity of casein fractions depends on the source of the enzyme. While α_s-casein

is less sensitive to factor XIIIa (animal tissue: Traore and Meunir, 1991; Ikura et al. 1980), it is highly sensitive to the attack of transglutaminase from *Streptoverticillium* sp. (microbial source: Ikura et. al., 1992; Christensen et al., 1996). Acyl donor sites of guinea pig liver transglutaminase in casein fractions (α_s, β, κ) were previously detected by Christensen et al. (1996).

The formation of heterologous dimers and polymers between different proteins was studied by Han and Damodaran (1996). They found that cross-linking dissimilar proteins such as caseins and albumins was not possible, whereas cross-linking similar proteins such as albumins, globulins, or caseins can occur. In addition, caseins have been used in several assays for the determination of transglutaminase activity and for analyzing enzyme specificity (Lorenzen et al., 1998).

TG can act on milk casein micelles by introducing intramolecular bonds to micelle cores, rendering micelles more stable under different treatments (i.e., acidification and cooling) (Takeuchi & Cunha, 2008) due the formation of cross-links between denatured whey proteins from heat-prepared milk and caseins. Secondly, by cross-linking the proteins at the micelle surface, the strength of acid gels formed by cross-linked micelles is increased. Concurrently, pores in the protein network become much smaller, providing improved moisture retention and stability (Takeuchi & Cunha, 2008). Spontaneous syneresis of water from acidified milk gels represents a problem especially in low-fat systems. Studies with acidified sodium caseinate gels suggest that only a small number of cross-linking sites are necessary for complete oligomerization of caseinates (Lorenzen et al., 1998), and that the smoothness and porosity of the acidified caseinate gels are extremely sensitive to the presence of TG (Partanen et al., 2008).

As most conventional food products contain more than 50% water, good water-holding capacity is essential because consumers tend to avoid products that show free water in the package (Barbut, 1996). Knowledge of the system's rheological or mechanical properties is of great importance, as texture attributes are significantly correlated with these properties. However, in order to make an adequate correlation among such properties, it is important to first characterize the microstructure of the system. Microstructures of gels are commonly studied using conventional and confocal light microscopy, but for structures smaller than 1 μm, scanning electron microscopy (SEM) or transmission electron microscopy is used. SEM is a good technique for imaging three-dimensional objects (Kalab, 1993), depicting morphological (i.e., number and size) and topographical features (structure discontinuity, pores or holes) with very good spatial resolution, which can be used to visualize or verify structural differences between samples. Such differences could then be correlated with mechanical or water retention properties of the system. Thus, the aim of the present work was to study the effect of TG from the newly isolated *Streptomyces* sp. CBMAI 837, and the consequent induction of cross-linking reactions among protein segments of acid caseinate gels on the microstructure, water-holding capacity, and mechanical properties of the obtained sodium caseinate gels. For this purpose, we compared our results with those of systems produced with a commercial enzyme Activa® TG-BP from Ajinomoto Co.

MATERIALS AND METHODS

Materials

Sodium caseinate HV was purchased from Merck (Darmstadt, Germany), D-glucono-δ-lactone (GDL, G4750) from Sigma Chemicals (St. Louis, MO, USA), and the commercial Ca^{2+}-independent TG product Activa® TG-BP was provided by Ajinomoto Inc. (Tokyo, Japan).

Transglutaminase and analytical methods

TG from *Streptomyces* sp. CBMAI 837 was obtained as described by Macedo et al. (2008) and was used without further purification. The commercial TG Activa® TG-BP contained the following excipients: 60% sodium caseinate, 5% lipid sucrose ester, and 34.4% dextrin. Transglutaminase activity was determined by hydroxamate formation with the specific substrate N-carbobenzoxy-L-glutaminyl glycine (N-CBZ-Gln-Gly) according to Folk and Cole (1966) with some modifications (Macedo et al., 2008). The measured activities of the commercial TG enzyme powder and the *Streptomyces* sp. CBMAI 837 enzyme were $1.53.10^{-2}$ U/mg and $1.58.10^{-2}$ U/mg, respectively. For that, both powder enzymes, on the mixture composition obtained as cited above, were considered to have the same enzymatic activity for mass unit at neutral pH.

Preparation of sodium caseinate gels

Solutions containing 4% and 8% sodium caseinate in distilled water were mixed with a magnetic stirrer for 30 min at room temperature, acidified with 0.8% and 1.6% GDL, respectively, and mixed for additional 15 min. The GDL/caseinate ratio was kept constant at 0.2 (Braga et al., 2006).

After addition of GDL, a 2% w/w amount of commercial TG and TG from *Streptomyces* sp. CBMAI 837 preparations containing similar TG activities were added to 25 mL aliquots of sodium caseinate solutions and mixed for 10 min. Those gels with added commercial transglutaminase were appropriately calculated to form 4% and 8% caseinate mass gels in order to maintain the consistency of the final percentages of caseinate on the ready gel, considering the caseinate content on the commercial enzyme samples as excipient. Samples without enzyme were also prepared. The solutions were poured into cylindrical plastic tubes (30 mm in diameter and 30 mm in height) for gel formation at room temperature for 24 h. The pH of the gels was monitored during the entire process. Decreases in pH from GDL hydrolysis was measured using a Sentron 2001 pH meter (Sentron Inc., Gig Harbor/Washington, USA) equipped with an electrode calibrated at the reaction temperature over the pH range from 4.0 to 7.0.

Protein electrophoresis

Caseinate gels were prepared as described above. After 24 h of acidification at room temperature (controlled at 22°C), the samples were frozen and freeze-dried. The samples were then dissolved in loading buffer (Tris-HCl with β-mercaptoethanol and SDS) at a concentration of 10 mg/mL, and boiled for 5 min at 95°C. The gel samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Vertical Slab Mini-Protean Electrophoresis System Bio-Rad Laboratories, Hercules, CA, USA) according to Laemmli (1970). The proteins were stained with a 0.1% solution of Coomassie brilliant blue R-250. Control references were as follows: (1) standard Low Molecular Weight (LMW) electrophoresis calibration kit (Pharmacia Biotech), (2) sample of the commercial enzyme and (3) sample of the TG from

Streptomyces sp. CBMAI 837. Both control references of the enzymes were dissolved in loading buffer at concentration of 10mg/mL, and boiled for 5 min at 95°C.

Water-holding capacity (WHC)

The gel cylinder (30 mm in diameter and 30 mm in height) was equilibrated at room temperature and cut into two gel discs of 5.0 ± 0.2 g. Each disc was placed into a 50 mL centrifuge tube. Water loss was determined in triplicate by weighing the water released after centrifugation at 173 x *g* for 10 min (Braga et al., 2006). WHC values were calculated using Eq. (1):

$$\text{WHC}\ (\%) = 100\left[1 - \left(\frac{Water_{loss}(g)}{Water_{gel}(g)}\right)\right], \quad (1)$$

where $\text{Water}_{\text{gel}}$ is the amount of water in the gel before centrifugation. The water loss of each sample was tested in duplicates.

Mechanical properties

Uniaxial compression experiments were performed using a TAXT Plus Texture Analyzer (Stable Microsystems Ltd., Godalming, UK) equipped with a lubricated acrylic cylindrical plate (60 mm diameter) as a probe. The gels were compressed to 80% of their original height using a crosshead speed of 1 $mm.s^{-1}$. All measurements were performed with five replications at 10 ± 1°C. The force and height data were transformed into Hencky stress (σ_H)-Hencky strain (ε_H) curves (Steffe, 1996). Rupture properties (stress and strain) were obtained from the maximum point of the stress-strain curve, while the elasticity modulus was obtained from the slope of the initial linear region of the curve. Stress (σ_{rup}) at rupture was used as indicator of gel hardness or firmness and the elasticity

modulus an indicator of gel strength, while the strain (ε_{rup}) at rupture measured gel brittleness (Kohyama and Nishinari, 1993).

Scanning electron microscopy (SEM)

Gel samples (10 mm x 3 mm x 1 mm) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 overnight. After two rinses of cacodylate buffer, the samples were fractured under liquid nitrogen and post-fixed in 1% buffered osmium tetroxide for 2.5 hours. The fixed samples were rinsed again three times with cacodylate buffer. The fractured gels were then dehydrated in a graded ethanol series (30%, 50%, 70% and 90%). Dehydration was continued in 100% ethanol (three changes over 1 h) followed by critical point drying (Critical Point Dryer CPD03 Balzers, Alzenau, Germany). The dried samples were mounted on aluminum stubs and coated with gold in a Sputter Coater SCD 050-Balzers (Alzenau, Germany). At least five images of typical structures at 500X magnification were obtained. Images were captured using a JEOL JSM 5800 LV (Tokyo, Japan) operated at 10 kV.

Statistical analysis

Values reported for mechanical properties (stress at rupture, elasticity modulus, and strain at rupture) represent the mean from five replicates obtained for at least two repetitions of each treatment; error bars represent one standard deviation. The SEM images represent typical structures obtained from at least five replicates for a minimum of two repetitions of each treatment. Significant differences ($p < 0.05$) between gels properties were determined by analysis of variance (ANOVA), and comparisons between

the mean values were evaluated by the Tukey procedure. Statistical analyses were performed using the STATISTICA 5.5 software (Statisoft Inc., Tulsa, USA).

RESULTS

Electrophoresis

Figure 4.1 shows the SDS-PAGE of proteins from sodium caseinate gels prepared with commercial TG (lanes C and D) and *Streptomyces* sp. CBMAI 837 TG (lanes E and F).

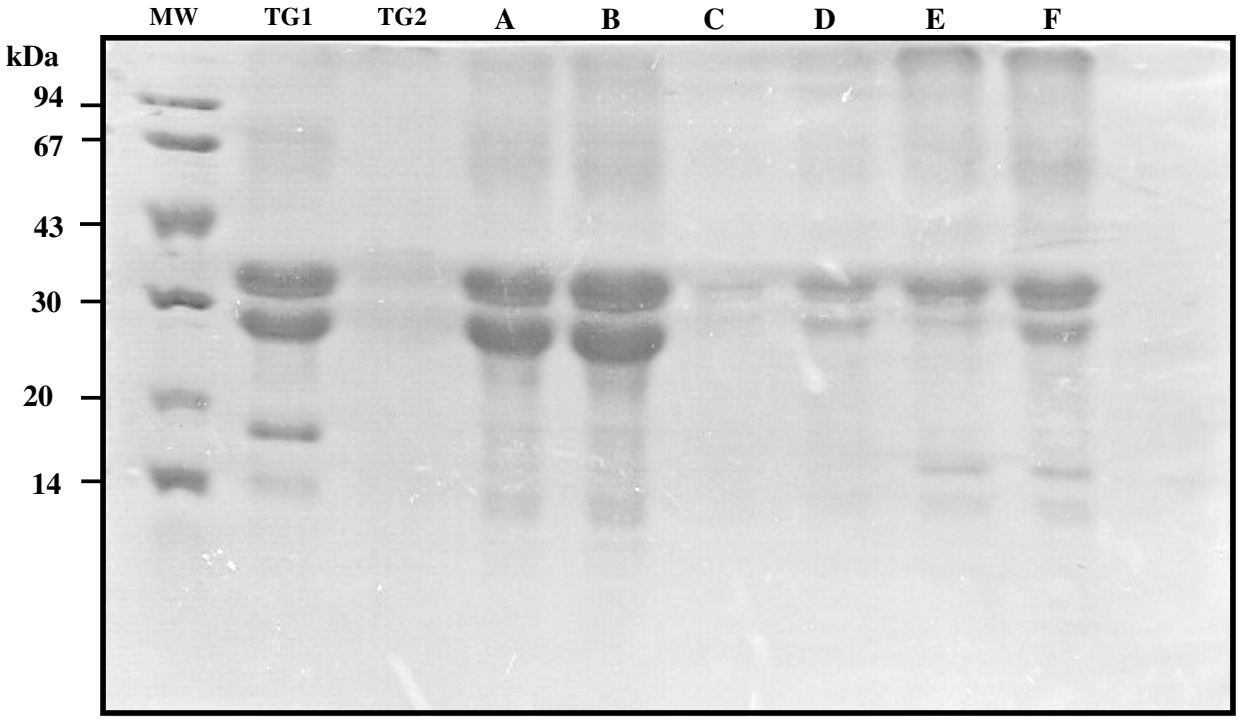

Figure 4.1. SDS-PAGE of proteins of sodium caseinate gels acidified with GDL and prepared with or without TG.

Lane	(% w/w) caseinate	(% w/w) TG
A	4%	0%
B	8%	0%
C	4%	2% Commercial
D	8%	2% Commercial
E	4%	2% CBMAI 837
F	8%	2% CBMAI 837

Reference controls

MW	Standard low molecular mass marker
TG1	Commercial TG
TG2	TG from *Streptomyces* sp. CBMAI 837

Lanes TG1 and TG2 correspond to the preparation of commercial TG and from *Streptomyces* sp. CBMAI 837, respectively. TG1 showed three pronounced bands with molecular masses above 30 kDa, between 20 and 30 kDa, and between 14 and 20 kDa. The molecular weights of some microbial transglutaminases include 29 kDa from *Bacillus subtilis* (Suzuki et al., 2000), 38 kDa from *Streptoverticilium* (Ando et al., 1989), and 45 kDa from *Bacillus circulans* (Soares et al., 2003). However, no clear bands were

observed between 14-94 kDa for TG2, indicating that the enzyme concentration was not high enough to evaluate its molecular weight. Thus, bands for TG1 may be associated with the presence of caseinate as an enzyme excipient. Caseins generally exhibit molecular masses in the range of 19 to 25 kDa, but molecular masses higher than 30 kDa were observed in our SDS-PAGE. This unexpected result can be due to competing equilibrium, as caseins either bind SDS or interact with other casein molecules in solution (Basch et al., 1985).

From Figure 4.1, it is possible to conclude that gels with no TG added showed very similar protein profiles and much stronger bands of casein fractions than gels with TG added. However, lane B showed more pronounced bands than lane A, possibly due to the higher protein concentration in gel B. Addition of TG decreased the intensities of the 27 kDa and 33 kDa protein fractions, indicating that the enzyme promoted bonding of these fractions into larger protein polymers. An increase in protein fractions with higher molecular mass (> 94 kDa) (lines E and F) as well as an increase in the 15 kDa protein fraction was observed when using TG from *Streptomyces* sp. CBMAI 837, which could correspond to free κ-casein. On the other hand, the gel containing the commercial enzyme (lanes C and D) showed smaller amounts of protein in the range between 14 and 94 kDa, suggesting that the commercial enzyme promotes formation of even higher molecular mass protein polymers (higher than 94 kDa) than that of the CBMAI 837 TG. If the commercial TG is producing higher molecular mass protein polymers, then these proteins should be retained in the 5% stacking gel (Fig. 4.1). These observations led to the hypothesis that the commercial enzyme rather than the TG from *Streptomyces* sp. CBMAI 837 is acting in this system to promote higher molecular mass protein polymers.

Water-holding capacity (WHC)

Results for the water-holding capacity (WHC) of the gels are shown in Figure 4.2.

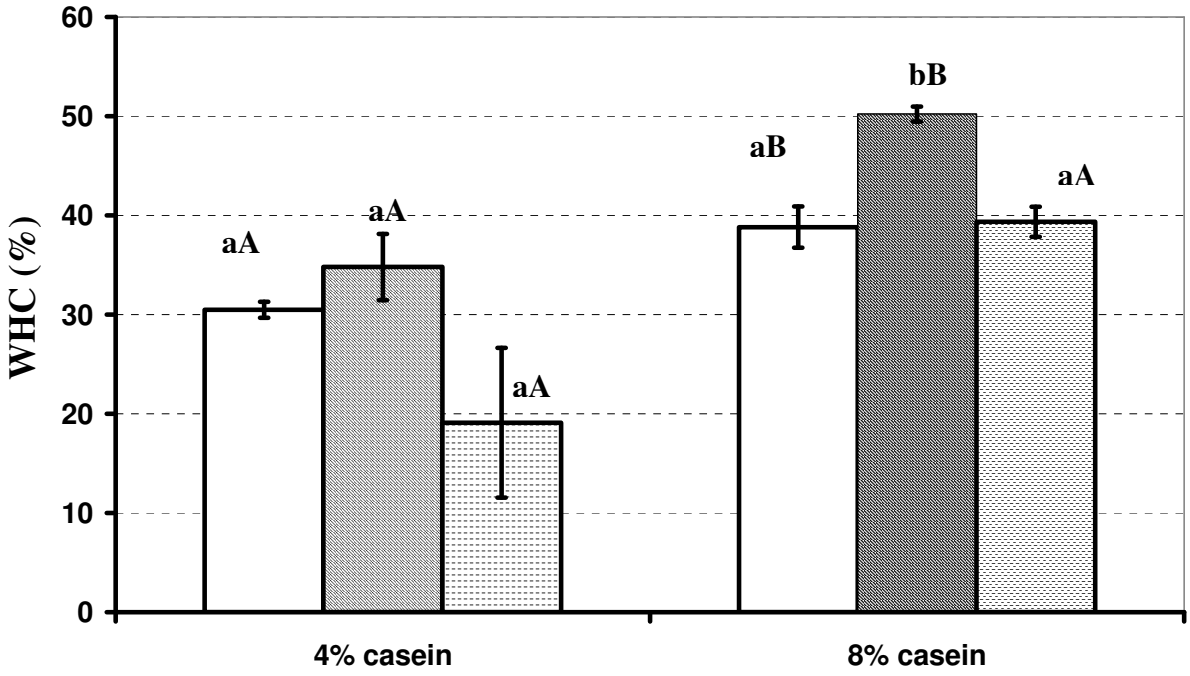

Figure 4.2. Water-holding capacity (%) of 4% and 8% acidified sodium caseinate gels with the addition of different TGs. Gel systems: ☐ 0% TG, ▨ 2% commercial TG and ▤ 2% TG from *Streptomyces* sp. CBMAI 837. Error bars represent standard deviation between replicates. Different letters designate significant differences (p<0.05). Small letters: differences among the type of TG added to each sodium caseinate concentration. Capital letters: differences among sodium caseinate concentration in each type of TG added.

Higher water-holding capacity (WHC) values were generally observed for gel samples with 8% caseinate than for samples with 4% caseinate (Fig. 4.2), a recurrent observation in casein or whey protein gels (Braga and Cunha, 2005; Cavallieri et al., 2007). This type of behavior is attributed to the increased number of hydrophilic sites present on protein structures, promoting interactions with free water molecules. The

presence or absence of any TG tested did not significantly influence the WHC results except for the 8% caseinate gel with commercial TG, which showed significantly higher values of WHC than other samples with the same caseinate concentration.

Mechanical properties

All caseinate solutions achieved a pH 4.6 ± 0.1 after 24 h of storage at room temperature with a caseinate/GDL ratio of 0.2. As this is approximately the pI of sodium caseinate (Takeuchi and Cunha, 2008), all the solutions formed self-supported gels allowing compression measurements. Figures 4.3A and 4.3B show the obtained stress and strain at rupture, respectively. An increase in sodium caseinate concentration in gels without TG led to a small increase in values of stress at rupture (Fig. 4.3A and 4.3B). In general, addition of TG led to a greater increase in stress and strain at rupture with a more pronounced increase at higher sodium caseinate concentrations. Addition of the enzyme to 8% (w/w) sodium caseinate gels led to nearly double the values for stress at rupture than that observed for 4% (w/w) sodium caseinate gels. Concerning the type of TG added to the system, it is interesting to observe that different behaviors on stress at rupture were observed with respect to protein concentration. The addition of commercial TG on 4% sodium caseinate gels led to more rigid gels than those obtained with TG from *Streptomyces* sp. CBMAI 837 (Fig. 4.3A). However, for the 8% sodium caseinate gel the values of stress at ruptures were similar for both types of enzymes added in the systems. This pattern was different for the strain at fracture (Figure 4.3 B), since the gel produced with CBMAI 837 showed a greater increase of strain at rupture in relation to gels produced with commercial TG.

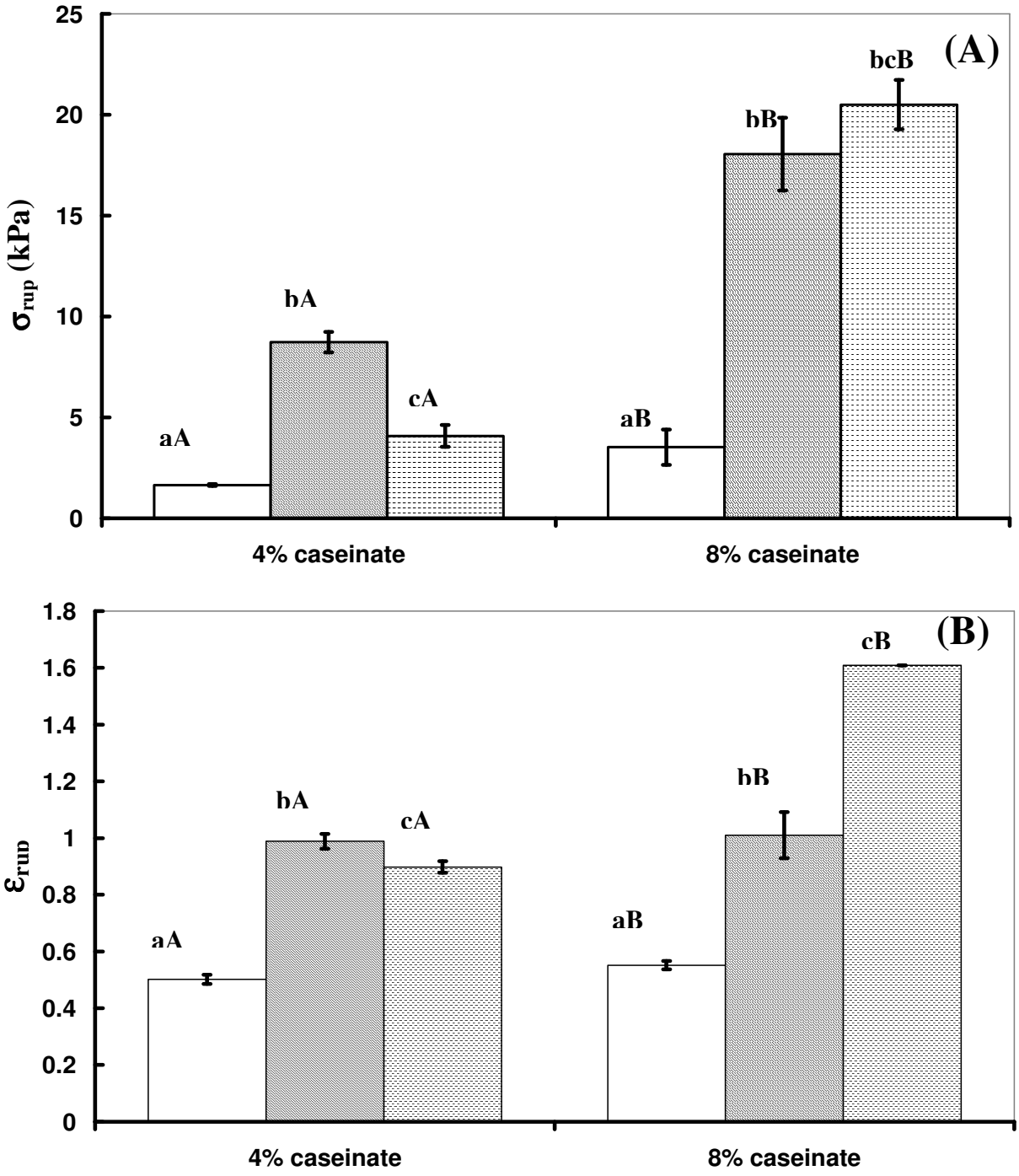

Figure 4.3. Stress at rupture (σ_{rup}) (A) and strain at rupture (ε_{rup}) (B) of 4% and 8% acidified sodium caseinate gels with the addition of different TGs. Gels systems: □ 0% TG, ▨ 2% commercial TG and ▨ 2% TG CBMAI 837. Error bars represent standard deviation between replicates. Different letters designate significant differences (p<0.05). Small letters: differences among the type of TG added to each sodium caseinate concentration. Capital letters: differences among sodium caseinate concentration in each type of TG added.

The results of the elasticity modulus (E_H) determined from initial raw stress-strain data (up to 10% of initial gel height) are shown in Figure 4.4. For 4% sodium caseinate gels, the elasticity modulus behavior was very similar to that observed for stress at rupture with respect to addition of the enzyme, but such patterns were not similar for higher sodium caseinate concentrations. The increase in sodium caseinate concentration led to a greater increase in E_H values, and such values were even greater for commercial TG gels. On the other hand, addition of TG from *Streptomyces* sp. CBMAI 837 led to a decrease in E_H values compared to the non-TG gel samples.

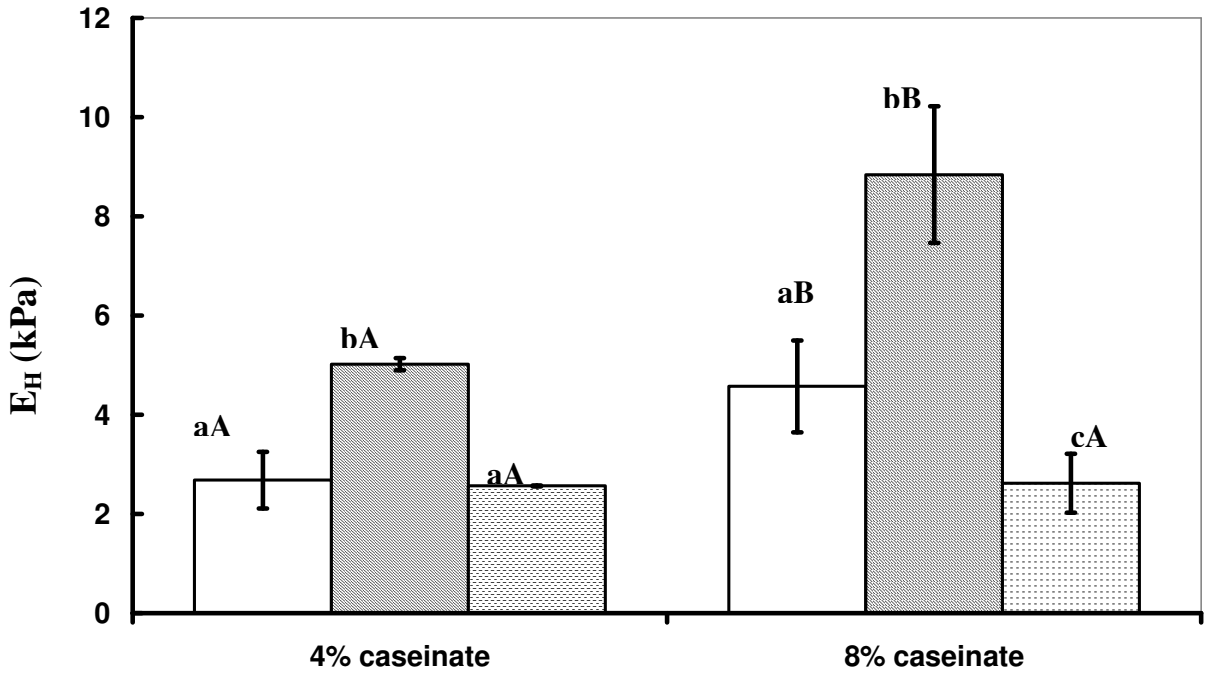

Figure 4.4. Elasticity modulus (E_H) of 4% and 8% acidified sodium caseinate gels with the addition of different TGs. Gel systems: ☐ 0% TG, ▨ 2% commercial TG and ▤ 2% TG from *Streptomyces* sp. CBMAI 837. Error bars represent standard deviation between replicates. Different letters designate significant differences (p<0.05). Small letters: differences among the type of TG added to each sodium caseinate concentration. Capital letters: differences among sodium caseinate concentration in each type of TG added.

During the slow acidification promoted by GDL hydrolysis, proteins are known to first aggregate into particles, and then later fuse into rough homogeneous strands (Braga et al., 2006). It is interesting to observe that biochemical characterization studies of transglutaminases (both commercial and crude TG from *Streptomyces* sp.) show that at pH values around 4.5 (the pH value of the gel at the end of the GDL acidification process), there is a strong decrease in transglutaminase activity, particularly for the *Streptomyces* sp. enzyme. Further biochemical characterization of transglutaminase is described in chapter 3. Thus, TG must be present at the beginning of the gel formation process to provide the enzyme the time and pH conditions needed to catalyze bonding reactions. In this work, the addition of an acid precursor (GDL) to the enzyme was carried out at the same time at room temperature, which caused a slow decrease in pH up to the measured gel firmness after 24 h, producing a more pronounced enzyme action at the beginning of the acidification process. All gels formed were self-supported, which could be tested and compared in mechanical attributes. The obtained results suggest that the increase in protein concentration of only acidified systems has a small effect on gel rigidity and practically no effect on gel deformability (Figs. 4.3A and 4.3B, respectively). However, increases in the elasticity modulus (Fig. 4.4) is related to enhancement in protein interactions during acidification, which could be associated to the increased number of charged residues able to interact at higher protein concentration.

Addition of TG to the gel systems led to stronger and more deformable gels (greater stress and strain values), once the TG promoted covalent bonds between protein molecules. The increase of these characteristics was proportional to the sodium caseinate concentration or the enzymatic reaction substrate concentration (Figures 4.3A and 4.3B).

However, the significant difference in the elasticity modulus obtained with each of the enzymes tested (Fig. 4.4) suggests that the reaction mechanisms of the commercial TG and TG from *Streptomyces* sp. CBMAI 837 may be very different, especially concerning short-range electrostatic or hydrophobic interactions among protein aggregates in the gel.

Microstructure of gels

All gel samples contained either 4% or 8% (w/w) sodium caseinate and were made in the presence of two different TGs, as well as samples without the enzyme (Figure 4.5 micrographs). There are several striking differences between the microstructures presented in Figure 4.5, demonstrating the effects of increasing concentrations of sodium caseinate as well as the mode of action of the two TGs tested.

The acidified casein gels consisted of a coarse particulate network of casein particles linked together in clusters, chains, and strands, as reported by Kalab et al. (1983). An increase in sodium caseinate concentration resulted in an increase of pore size in gel samples without TG, and a more particulate structure was observed in the gel network. When the pH value was lowered toward the pI, neutralization of the negative charge caused a decrease in electrostatic repulsion (Walstra, 1990), promoting more intensive protein-protein interactions and changing the organization and strand formation in the casein gel network.

Addition of TG modified the microstructure of the sodium caseinate gels (Figures 4.5C to 4.5F), while addition of commercial TG (Figures 4.5C and 4.5D) led to regions with a more compact and interconnected structure and smaller pores than gels without TG. The 4% w/w sodium caseinate gel prepared with commercial TG (Fig. 4.5C) showed

a more particulate structure than 8% sodium caseinate gels with commercial TG (Fig. 4.5D).

On the other hand, addition of TG from *Streptomyces* sp. CBMAI 837 led to gel structures with larger pores and different patterns of strand formation (Figures 4.5E and 4.5F). However, the gel prepared with 8% sodium caseinate and TG from *Streptomyces* sp. CBMAI 837 (Fig. 4.5F) showed a net structure more homogeneous and cohesive than those prepared without TG (Fig.4.5B). Thus, TG may induce cross-links that cause reorganization of surface aggregates (charge and structure), more easily allowing gel formation to occur.

The more compact structure of gels prepared with commercial TG was observed in the greater E_H values (Fig. 4.4) and the intensified cross-links between protein segments in the electrophoresis results (Fig. 4.1, lanes C and D). On the other hand, the structure of gels prepared with TG from *Streptomyces* sp. CBMAI 837 was related to the lower molecular mass protein polymers formed by this enzyme. The smaller protein polymers in the structure could be associated with differences between these two enzymes and the relative activity decrease with pH decay. Previous studies showed that the transglutaminase activities of the commercial TG and TG from *Streptomyces* sp. CBMAI 837 were affected differently by decreases in pH. From neutral pH to caseinate pI (4.6), the enzyme activities fall to 55-50% on the commercial enzyme and nearly to 10%, on the TG from *Streptomyces* sp. CBMAI 837 (Macedo et al., 2008)(chapter 3). These microstructures observation could explain the observation of gels with increased stress and strain at rupture (Figs. 4.3A and 4.3B).

Differences among mechanical properties (stress and strain at fracture or elasticity modulus) can be related to varying aspects of gel structure including the scale of deformation applied to determine mechanical properties. The stress/strain at rupture are parameters determined at higher deformation levels (at 80% of the initial gel height), and are therefore related to the structure and organization of the whole gel. On the other hand, the elasticity modulus is a parameter determined at small deformation (10% of the initial gel height) and is more accurately related to interactions among protein segments in the gel structure. As a result, the gels prepared with commercial TG gels showed greater values of elasticity modulus, indicating that this parameter could be associated to cross-linking interactions promoted by addition of this enzyme, as observed in the electrophoresis results (Fig. 4.1). In this way, an important characteristic of the *Streptomyces* sp. CBMAI 837 TG is that the addition of such an enzyme could lead to the formation of more rigid but less elastic gels, while commercial TG promotes the formation of more elastic and soft food systems.

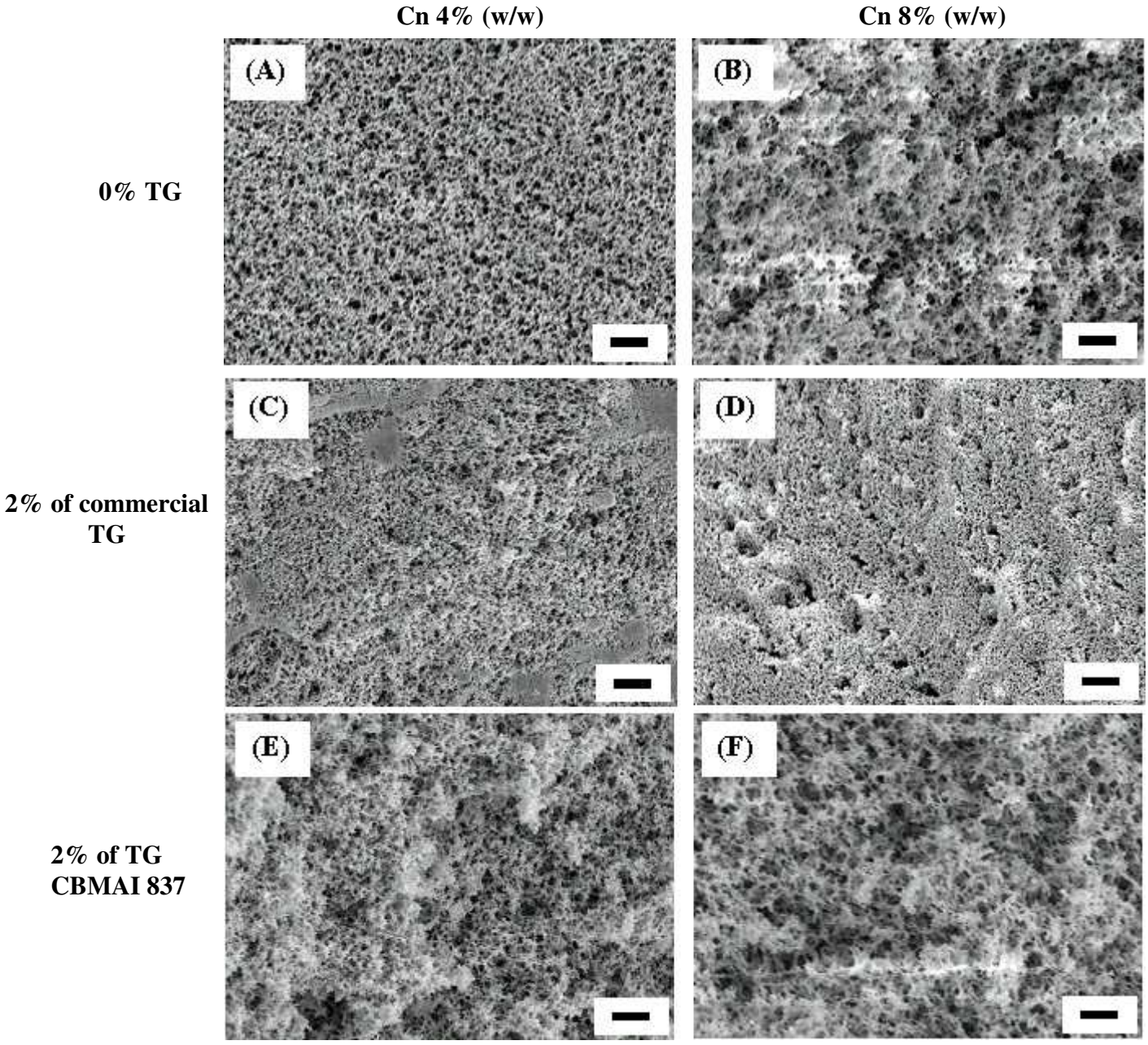
Cn 4% (w/w)
Cn 8% (w/w)
0% TG
2% of commercial TG
2% of TG CBMAI 837
(A)
(B)
(C)
(D)
(E)
(F)

Figure 4.5. SEM micrographs of acidified sodium caseinate (Cn) gels (4% w/w, left column; 8% w/w, right column) prepared with or without TG. (A, B) No addition of TG; (C, D) 2% commercial TG and (E, F) 2% TG from *Streptomyces* sp. CBMAI 837. Scale bars correspond to 10 μm.

CONCLUSION

Caseinate gels prepared with the commercial TG and TG from CBMAI 837 led to different gel networks and properties due to the particular action of each enzyme. Addition of *Streptomyces* sp. CBMAI 837 TG led to the formation of more rigid and deformable but less elastic gels, while addition of the commercial TG promoted formation of more elastic and soft food systems. Moreover, it was possible to observe how enzymes from different microbial sources can show differences in action when applied to the same food systems. These observations could be of interest in future food design and open up opportunities to formulate food products or even food ingredients with specific texture attributes depending on the specific cross-link reactions between protein segments in the food product.

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CAPÍTULO 5. TAXONOMIC INVESTIGATION OF THE TRANSGLUTAMINASE PRODUCER ACTINOMYCETE STRAIN P20

Juliana Alves Macedo, Lara Durães Sette[2], Hélia Harumi Sato[1]

ABSTRACT

An actimonycete strain isolated from Brazilian soil presented a significant transglutaminase production. This strain, named P20, was taxonomically investigated in the present work by using a combination of molecular and morphological based-methods. Data derived from phylogenetic analysis showed that strain P20 belongs to the genus *Streptomyces*, which is closely related to the *Streptomyces hygroscopicus* NBRC13786, *Streptomyces platensis* NBRC12901, and *Streptomyces caniferus* NBRC15389 type strains. Considering the molecular, morphological, and physiological results together, strain P20 is most closely related to *S. platensis*. However, there are some differences in the cultural and physiological characteristics. Even though a definitive taxonomic assignment of strain P20 characterized in this study was not possible, little is known about *S. platensis* transglutaminase characteristics and, additionally, the ability of *S. caniferus* to produce transglutaminase is not actually revealed. In this context, strain P20 can be considered as a great target for further investigations concerning transglutaminase production.

Keywords: actinomycete, molecular taxonomy, conventional taxonomy, polyphasic taxonomy, transglutaminase

INTRODUCTION

Actinomycetes are widely distributed in terrestrial environments with high abundance and metabolic divergence. The greatest population density of this group of microorganisms is found in soil. Numerically, they are less dominant than the other bacteria and more prominent than fungi (Arai, 1997; Sette et al., 2005).

The actinomycetes have long been isolated and studied as a source of commercially useful enzymes and others bioactive molecules. Transglutaminase (EC 2.3.2.13; protein-glutamine y-glutaminyltransferase) is one important enzyme in the food-processing industry because of its ability to catalyze reactions for the creation of new product textures, the modification of viscosity, the alteration of emulsifying and foaming properties, and the improvement of product nutritional value (Zhu et al. 1995; Kwan and Easa, 2003). The first microbial transglutaminase characterized was from actinomycetes (Ando et al. 1989) and since then, efforts have been made to obtain mass production of this enzyme for commercial applications, especially for the enzymes from the genus *Streptomyces* (Gerber et al. 1994, Zhu et al. 1998, Zotzel et al., 2003).

The proper identification and classification of microorganisms is critical to the study of bioactive compound production. In most cases, the ability to produce one or another bioproduct is taxonomically determined. In addition, without proper identification and preservation of the isolates, chemical investigations become difficult, if not impossible, to reproduce. Traditionally, identification of bacteria, including actinomycetes, has been very time consuming and laborious, except for some pathogenic

species for which simple identification methods have been established (Muramatsu, 2008). Although actinomycetes have historically been identified and classified primarily by morphological characteristics, taxonomists now employ a number of techniques to help in the actinomycetes' identification and to organize their systematics. Even so, the molecular-based methods have provided a rapid and accurate way to identify bacteria, including the actinomycetes (Cook and Meyers, 2003); for a good characterization and identification system, a polyphasic approach has to be performed by using the molecular and morphological based-techniques together.

In this study, the 16S rDNA sequenced-based method and general morphological and biochemical properties of the actinomycetes were employed for the taxonomic characterization of transglutaminase producer strain P20.

MATERIALS AND METHODS

Strain

Actinomycete strain P20 used in this study was isolated from soil sample collected from the State of São Paulo in Brazil, as described by Macedo et al. (2007). This strain was selected for presenting the strongest transglutaminase activity in laboratory conditions (Macedo et al., 2007; Macedo et al., 2008) (chapter 2) and was preserved in glycerol solution at -80 ºC. After identification, the strain was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI), filed under access number CBMAI 837.

Morphological and physiological characterization of strain

Characterization of strain P20 was performed according to Shirling and Gottlieb (1966), with cultivation in different growth media (7 to 14 days, 30 ºC) to determine: (1) the morphological characteristics of spore-bearing hyphae, spore chain morphology, and surface ornamentation (International *Streptomyces* Project, ISP 3: Oatmeal agar); (2) the color of spore mass, substrate mycelium, and diffusible soluble pigments (ISP 4: inorganic salts-starch agar, and ISP 5: glycerol–asparagine agar); and, (3) melanin production (ISP 6: peptone iron agar supplemented with 0.1% yeast extract, and ISP 7: tryptone–yeast extract broth). Spore chain morphology was determined by direct light microscopic (LM) examination of the culture surface (ISP 3: Oatmeal agar) using the Leica MZ6 equipment. Scanning electron microscopy (SEM) was done according to standard protocols used at EMBRAPA-CNPMA (Jaguariúna, SP, Brazil) as described by Sette, et al. (2005) in a Carl Zeiss/Leica model LEO 982 electron microscope.

The carbon utilization tests were performed as described by Shirling and Gottlieb (1966). The appropriated washed inoculums were inoculated in plates that contained different carbon sources. The plates were prepared first with a sterile basal medium ($(NH_4)_2SO_4$: 2.64g; KH_2PO_4: 2.38g; K_2HPO_4. $3H_2O$: 5.65g; $MgSO_4.7H_2O$: 1.00g; distilled water: 1L), and then the carbon source solution (1%), sterilized by filter sterilization, was added to the Petri dish. The carbon sources tested were: arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose. Approximately 0.05 mL of the washed inoculums was inoculated at each plate and the plates were incubated at 30°C, and the growth of the strain was observed after 5 and 15 days. All tests were done in duplicates.

16S rDNA isolation, amplification and sequencing

For the 16S rDNA sequence analyses, genomic DNA was extracted from cultures using Pitcher et al. (1989) protocol, with minor modifications as described by Sette et al. (2005). PCR amplification of 16S rDNA was done by using the bacterial primer set p27f (5´ AGA GTT TGA TCM TGG CTC AG 3´) (Lane 1991) and p1401r (5´ CGG TGT GTA CAA GGC CCG GGA ACG 3´) (Heuer et al. 1997). The reactions (50 µL) contained 50-100 ng of genomic DNA, 2 U of *Taq* polymerase (GE Healthcare), 0.2 mmol L^{-1} of dNTP mix, and 0.2 µmol L^{-1} of each primer, in 1X *Taq* buffer. The PCR amplifications were done using an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 55 ºC, and 3 min at 72 ºC, and a final extension at 72 ºC for 5 min, in a *BIORAD* iCycler (Biorad®).

Amplified products were purified by using GFX PCR DNA and a gel band purification kit (GE Healthcare), quantified and subjected to sequencing using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for an automated MegaBase sequencer (GE Healthcare). The sequencing reactions (10 µL) contained 200 ng/µL of amplified DNA; 3.2 pmoles/µL of the primer and 4 µL of the kit DYEnamic ET Dye Terminator Cycle (GE Healthcare) and were submitted to the following amplification program: 30 cycles of 20s at 95 °C, 15s at 50 °C and 1 min at 60 °C in a *BIORAD* iCycler (Biorad®). The set of primers used for sequencing were 28f (5´ GAG TTT GAT CCT GGC TCA G 3`), 765f (5´ ATT AGA TAC CCT GGT AG 3´) (Stackebrandt and Charfreitag, 1990); 782r (5´ ACC AGG GTA TCT AAT CCT GT 3´) (Chun, 1995) and 1100r (5´ AGG GTT GGG GTG GTT G 3´) (Lane, 1991).

Phylogenetic analysis

Sequences were compared with 16S rRNA sequence data from type strains available at the public database Genbank (http://www.ncbi.nem.nih.gov) by using the BLAST N sequence match routines. The sequences were aligned using the CLUSTAL X program (Thompson et al. 1994), and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura et al. 2007). The Kimura two-parameter model (Kimura 1980) was used to estimate evolutionary distance. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

RESULTS

The actinomycete strain P20 from Brazilian soil that was selected to present a strong transglutaminase production was taxonomically investigated in the present work by using a polyphasic approach. Data from molecular and conventional analyses were used to characterize taxonomically the actinomycete strain isolated from Brazilian soil.

16S rDNA partial sequence of the strain was compared to sequences of organisms represented in the database Genbank and the results showed that actinomycete strain P20 belongs to the genus *Streptomyces* sp. (Table 5.1). Strain P20 showed 99.75% sequence similarity with the type strains of *Streptomyces platensis* NBRC 12901, *Streptomyces hygroscopicus* subsp. *glebosus* LMG 19950, *Streptomyces libani* subsp. *rufus* NBRC 15424 and *Streptomyces caniferus* NBRC 15389. The type strain's sequences were recovered from the databases and aligned with the 16S rDNA sequence of the actinomycete strain P20. The alignments (about 1230 nt) were used to calculate distance

matrices and generate phylogenetic tree (Figure 5.1), as described previously (Material and Methods, Phylogenetic Analysis).

Table 5.1. Closest relatives of actinomycete strain P20 based on BLAST analysis

CBMAI accession number	*Closest related Species*	*% similarity*
837	*Streptomyces platensis* NBRC 12901^T	99.75
	Streptomyces hygroscopicus subsp. *glebosus* LMG 19950^T	99.75
	Streptomyces libani subsp. *rufus* NBRC 15424^T	99.75
	Streptomyces caniferus NBRC 15389^T	99.75

Based on the phylogenetic tree (Figure 5.1), the strain P20 (CBMAI 847) is grouped with *Streptomyces caniferus* type strain and with the cluster formed by the type strains of *Streptomyces hygroscopicus* subsp. *glebosus*, *Streptomyces platensis* and *Streptomyces libani* (bootstrap value of 85%). According to Bergey's Manual of Determinative Bacteriology (Pridham and Tresner, 1974), *S. platensis* and *S. libani* are considered as subjective synonyms. *Streptomyces ramulosus* and *Streptomyces catenulae* type strains were used in the phylogenetic tree as an outgroup.

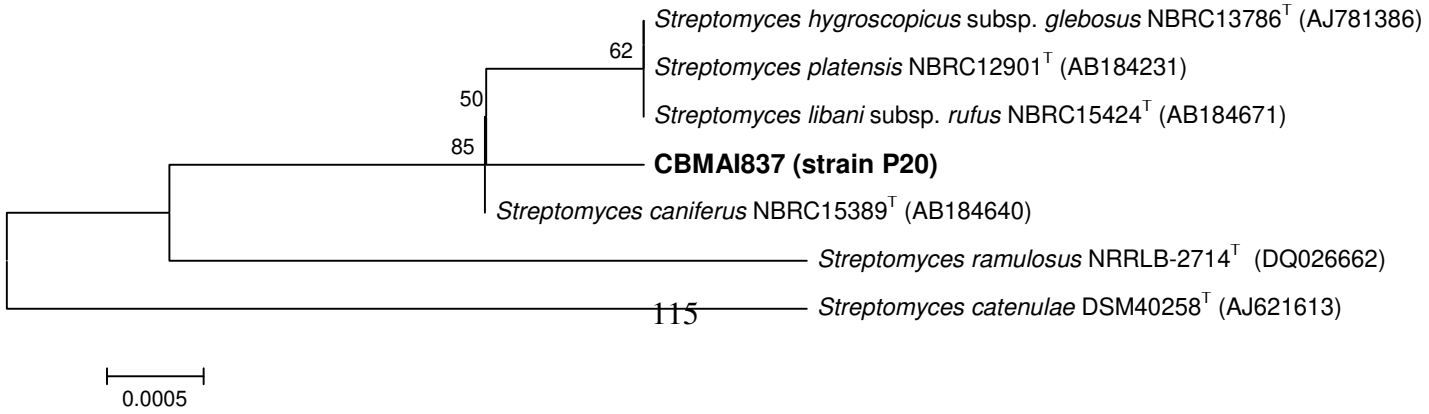


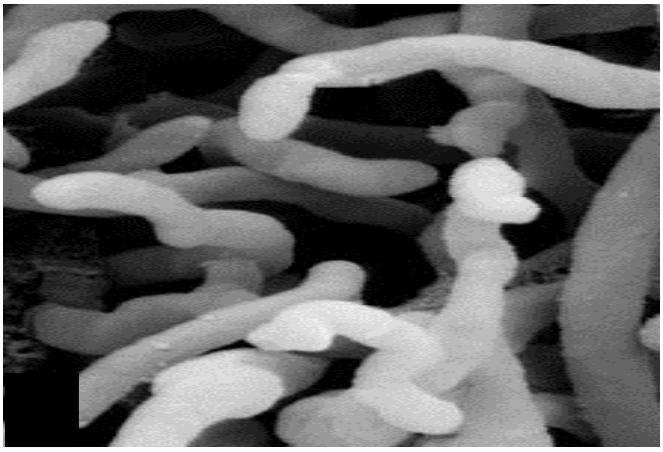
Figure 5.1. Phylogenetic tree based on 16S rDNA analyses, showing closest relatives of strain P20 (CBMAI 837) (Kimura two-parameter model; Neighbor-Joining algorithm and 1,000 replicate bootstrap).

Taxonomic characterization of the strain P20 was also done by using morphological and physiological data. The strain P20 presented morphological features that supported their assignment to the genus *Streptomyces*, namely highly-branched substrate mycelia with production of aerial hyphae and straight or spiral spore chains. Data derived from the predominant morphological and cultural features showed that strain P20 is very similar to *S. caniferus* and *S. platensis* with an open, loose, and stretched spiral spore chain, smooth spore surface ornamentation (Figure 5.2), gray spore mass (Figure 5.3) and reverse yellow-brown. However, strain P20 presented a melanin production in tryptone–yeast extract broth (ISP 7). According to the literature, melanoid pigment is not produced by *S. platensis* and this data was not found for *S. caniferus.* The differences between strain P20 and *S. hygroscopicus* are related to the rugose spore ornamentation and gray-black mass spore color formed by *S. hygroscopicus* when cultured in oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) media, respectively (Table 5.2).

Table 5.2. Morphological and physiological characteristics of the transglutaminase producer strain P20 (CBMAI 837).

Strain	*Spore chain morphology*[1]	*Spore surface ornamentation*[1]	*Spore color*[2]	*Reverse*	*Diffusible pigment*[2]	*Melanin on ISP6*	*ISP7*[*]
S. caniferus	Spirals	Smooth	Gray	Yellow	Absent/Yellow	NF	NF
S. platensis	Spirals	Smooth	Gray	Yellow-brown	Absent	-	-
S. hygroscopicus	Spirals	Rugose	Gray-Black	Yellow-brown	Absent	-	-
P20(CBMAI 837)	Spirals	Smooth	Gray	Yellow-brown	Absent	-	+

[1] ISP 3 media; [2] ISP 4 media; [3] ISP 5 media; * (+) present; (-) absent; (NF) data not found. Sources: Data from *S.platensis* (*S. libani*) and *S. hygroscopicus* were obtained from descriptions in the Bergey's Manual of Determinative Bacteriology (Pridham & Tresner, 1974). Data from *S. caniferus* were obtained from descriptions in the Gause et al. 1983.



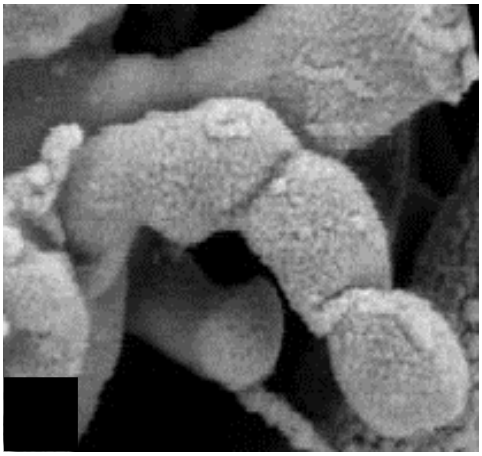


Figure 5.2. Scanning electron micrography of strain P20 (CBMAI 837)on ISP2 (0.4% yeast extract, 1% malt extract, 0.4% de glucose and agar, pH 7.0 ± 0.2) (incubated for 20 days at 30°C): A) spore chains morphology and, B) spore surface ornamentation

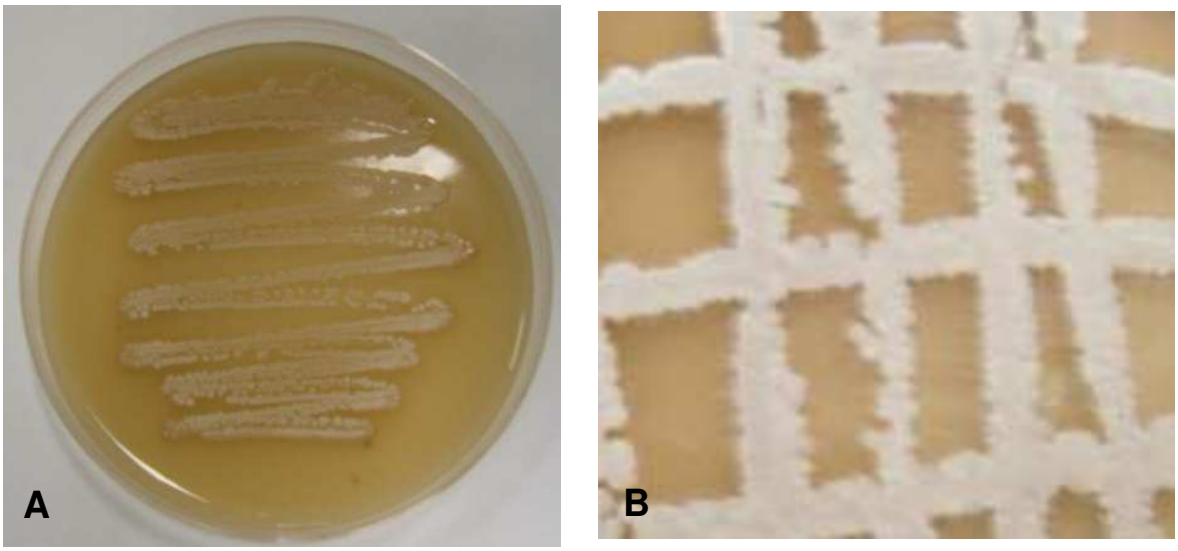


Figure 5.3. Strain P20 mass spore after growing on: A) oatmeal agar (ISP 3) and B) inorganic salts-starch agar (ISP 4) (incubated for 7 days at 30°C)

Additional carbon sources utilization experiments were conducted in order to help the elucidation of the strain P20 taxonomy. The pattern of carbon utilization is shown in Table 5.3. Strain P20 was able to utilize all carbon sources with the exception of rhamnose, arabinose, and xylose. In this experiment, strain P20 showed great similarity to *S. platensis* corroborating the data derived from morphological and physiological characteristics (Table 5.2), being different only in xylose utilization. Regarding carbon source utilization patterns between strain P20 and *S. caniferus*, these strains showed different results for rhamnose, fructose, and raffinose (Table 5.3).

Table 5.3. Carbohydrate utilization of strain P20 (CBMAI837), *S. platensis* and *S. caniferus*

Carbon sources	*CBMAI 837*	*S. platensis**	*S. caniferus***
Inositol	+	+	+
Mannitol	+	+	+
Rhamnose	-	±	+
Sucrose	+	+	+
Fructose	+	+	-
Arabinose	-	±	-
Xylose	-	+	-
Raffinose	+	+	-

*Sources: Bergey's manual and Iwami et al. (1983); **Source: Gause et al. (1983)
Symbols: (+) utilization; (±) doubtful utilization; (-) no utilization

DISCUSSION AND CONCLUSION

The above mentioned morphological/physiological characteristics and molecular analyses of strain P20 indicate that this strain belongs to the genus *Streptomyces*, but identification of isolates to the species level was not conclusive. Molecular and phylogenetic analysis showed that strain P20 is closely related to the following type strains: *Streptomyces hygroscopicus* subsp. *glebosus* NBRC13786, *Streptomyces*

platensis NBRC12901, *Streptomyces libani* subsp. *rufus* NBRC15424 and *Streptomyces caniferus* NBRC15389. The sequences similarity between strain P20 and the *Streptomyces* species cited above were 99.75%, and the genetic relationship was support by a great bootstrap value (85%).

Cultural and physiological characteristics and carbon utilization pattern of the strain P20 were compared with those of the species selected for a comparative study: *S. caniferus, S. platensis* (= *S. libani*) and *S. hygroscopicus*. Strain P20 was most similar to *Streptomyces platensis*, although there are some differences in cultural and physiological characteristics (see Tables 5.2 and 5.3).

Considering the molecular, morphological, and physiological results of comparative studies of strain P20 with the type cultures of the species *S. platensis*, *S. hygroscopicus* and *S. caniferus,* this strain is most closely related to *S. platensis*. However, there are some differences in the cultural and physiological characteristics between each other. Although those differences are not enough to establish a new species in the genus *Streptomyces*, the phylogenetic results grouped these two strains in a separated cluster (Figure 5.1) with high bootstrap support (85%). In this context and in order to get a more accurate taxonomic affiliation of the strain P20, new taxonomic experiments have to be performed, including rybotyping and DNA-DNA hybridization. These experiments will be conducted in collaboration with scientific research projects from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - DSMZ (German Collection of Microorganisms and Cell Cultures).

There are few studies on microbial transglutaminases. However, *Streptomyces* and *Streptomyces*-related strains such as: *Streptoverticillium griseocarneum*, *S. cinnamoneum*

subsp. *cinnamoneum* and *S. mobaraense* (Motoki et al. 1989), *Streptoverticillium* sp. (Ando et al. 1989), *Streptomyces hygroscopicus* (Cui et al., 2007), *Streptoverticillium ladakanum* (Ho et al., 2000) and *Streptomyces platensis* (Lin et al. 2006) are cited in the literature as transglutaminase producers. In the case of transglutaminase from *S. platensis* only one article was found in the consulted literature that considers cloning and expression of the gene coding for this enzyme in *S. lividans* (Lin et al., 2006). Information regarding enzyme characteristics was not cited by the authors.

Transglutaminases from *Streptomyces* are considered more advantageous than those from other species because of their extracellular formation and Ca^{2+}- independence (Liu et al., 2007). Though a definitive taxonomic assignment of the transglutaminase producer *Streptomyces* strain P20 (CBMAI 837) isolated from Brazilian soil and characterized in this study was not possible, there is no data in the consulted literature regarding the biochemical characteristics of transglutaminase from *S. platensis,* nor anything considering the ability of *S. caniferus* to produce this enzyme. Therefore, strain P20 could be considered as a great target for further enzymatic investigation.

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CONCLUSÕES GERAIS

Dentre as 200 culturas puras de actinomicetos isoladas e testadas para a produção de transglutaminase, a linhagem denominada P20 foi a selecionada, com uma atividade enzimática inicial de 0,25 U. mL^{-1}. A linhagem P20 foi identificada por métodos moleculares como *Streptomyces* sp. e está depositada na Coleção Brasileira de Culturas do Meio Ambiente e de Microrganismos Industriais (CBMAI), com o número de identificação CBMAI 837.

Utilizando metodologia de superfície de resposta, as melhores condições para a produção da transglutaminase foram: faixa de temperatura 25 – 30 ºC; 100-115 rpm; 2,5% farinha de soja; 0,4% KH_2PO_4; 0,2% $MgSO_4.7.H_2O$; 2,0% amido de batata; 0,1% glicose e 1,0% peptona bacteriológica. Utilizando esta metodologia, foi possível aumentar a produção da transglutaminase em cerca de 5,6 vezes (0,25 U. mL^{-1} para 1,4 U. mL^{-1}).

A etapa de purificação da transglutaminase foi realizada utilizando-se um processo rápido e simples, no qual uma solução da enzima bruta liofilizada foi aplicada em coluna de filtração em gel Sephadex G-75 duas vezes sucessivas. O fator de purificação atingido foi de 5,0 com 17% de recuperação. A purificação da proteína foi comprovada por homogeneidade eletroforética em SDS-PAGE. A massa molecular da enzima foi estimada em 45 kDa.

Nos estudos de caracterização bioquímica comparativos, a enzima da linhagem *Streptomyces* sp. CBMAI 837, bruta e purificada, apresentaram atividade ótima na faixa de pH entre 6,0-6,5. A enzima comercial apresentou os mesmos resultados. Um segundo ponto de pH ótimo de atividade enzimática foi observado no pH 10,0, tanto para a enzima

bruta, como pra comercial. Esse fato interessante nunca havia sido reportado na literatura antes. A temperatura ótima de atividade das amostras enzimáticas está em torno de 40-45°C. Todas as três preparações enzimáticas testadas mostraram-se estáveis na faixa de pH entre 4,5-8,0 e até 45°C.

Todas as transglutaminases testadas mostraram atividade enzimática independente da presença de Ca^{+2}, aumentada na presença dos íons K^{+}, Ba $^{2+}$, e Co $^{2+}$, e inibida por Cu^{2+} e Hg^{2+}, o que sugere a presença de um grupo tiol no sítio ativo da enzima. A enzima purificada apresentou K_m igual a 6,37 mM e V_{max} igual a 1,7 U/mL, enquanto a enzima bruta mostrou valores de K_m igual a 6,52 mM e um V_{max} de 1,35 U/mL.

Através do estudo de aplicação da enzima, foi possível verificar que a transglutaminase de *Streptomyces* sp. contribuiu para os parâmetros de textura dos géis de caseinato de sódio de forma diferente da enzima comercial, conferindo aos géis características próprias, tornando-se uma alternativa tecnológica para o desenvolvimento de produtos lácteos.

SUGESTÕES PARA TRABALHOS FUTUROS

1. Conduzir estudos para produzir a enzima utilizando escala piloto e industrial.
2. Conduzir estudos de produção da enzima por fermentação sólida
3. Testar outras formas de purificação da enzima, com uma recuperação maior na atividade enzimática.
4. Testar outras aplicações para a transglutaminase, tais como: aplicação em reestruturados de carne, microencapsulamento de óleos, ou enriquecimento de redes de glúten em farinhas pobres.
5. Testar técnicas de imobilização da enzima.

ANEXOS

O objetivo da inclusão dos anexos seguintes ao corpo deste trabalho é de registrar parte do trabalho experimental desenvolvido, mas que não está descrito nas publicações que formam os capítulos anteriores. Nestes anexos estão informações sobre como foram definidos os caminhos trilhados durante o trabalho de pesquisa, e resultados que não foram apresentados nos artigos científicos, mas que podem ter importância para definição de futuros projetos de pesquisa nessa área.

ANEXO I

Avaliação da produção de transglutaminase por fermentação semi-sólida

Algumas características de crescimento e produção da transglutaminase pelo *Streptomyces* sp. em estudo, sugeriram que talvez fosse viável realizar a fermentação para produção da enzima em meio semi-sólido. A aparente inadaptação do microrganismo ao crescimento em meio líquido, observada pela formação de "grumos" de massa celular durante a fermentação, e sua característica de crescimento formando pseudo-hifas, torna seu comportamento semelhante ao de fungos filamentosos. Alguns pesquisadores atribuem a capacidade desse gênero de microrganismo de produzir transglutaminase extracelular, a necessidade de estabilizar seu crescimento em forma de hifas (Yan et al., 2005). Todas essas observações levaram aos testes de fermentação em meio com alta densidade (descritos no capítulo 2) e também a um ensaio com fermentação semi-sólida.

Método

A produção de transglutaminase foi testada em frascos Erlenmeyer de 500 mL contendo 20 g do meio de cultura composto por farelo de trigo e água na proporção de 1:1 em massa, previamente esterilizados a 121º C e 1 atm de pressão por 20 minutos. Após incubação a 30 ºC durante 72 h ou até crescimento satisfatório, o meio foi triturado com 100 mL de H_2O destilada. Os frascos foram mantidos em agitação a 50 rpm a temperatura ambiente por 1h e a mistura foi filtrada em papel de filtro. A atividade de transglutaminase foi determinada, no filtrado, de acordo com as condições descritas anteriormente.

Resultados

Os testes realizados nas condições descritas na metodologia não resultaram em nenhuma atividade de transglutaminase. Dessa forma, esse caminho para produção da transglutaminase permaneceu inexplorado no restante deste trabalho. No entanto, existem estudos recentes (Nagy & Szakacs, 2008) que indicam que com uma seleção adequada do suporte para fermentação sólida, cepas produtoras de transglutaminase do gênero *Streptomyces*, são capazes de produzir a enzima por processos fermentativos em base sólida.

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ANEXO II

Estudo da semi-purificação e concentração da transglutaminase

O primeiro passo para o estudo da purificação do extrato bruto da transglutaminase de *Streptomyces* sp. foi a busca por um método adequado de concentração do sobrenadante da fermentação, que de preferência, resultasse em uma pré-purificação da enzima, facilitando sua posterior separação por métodos cromatográficos. Os ensaios realizados, antes da metodologia final (apresentada no capítulo 3) estão descritos neste anexo.

Método

A produção de transglutaminase foi realizada em frascos agitados em meio de cultivo e condições otimizadas determinadas como descrito na metodologia.

Foram testados os seguintes métodos de concentração da enzima bruta, a partir do sobrenadante da fermentação: (1) o fracionamento da transglutaminase do sobrenadante do meio de cultivo com sulfato de amônio nas concentrações de 40%, 60%, e 80% de saturação. Após cada etapa de saturação o precipitado foi separado por centrifugação a 10.000 x *g* por 10 min a 4ºC. O precipitado foi ressuspenso em água destilada e dialisado contra água destilada a 4ºC para a remoção do sal. Ao término da diálise, o extrato obtido foi congelado a -18°C. O sobrenadante foi saturado com sulfato de amônio e o processo repetido. As preparações brutas obtidas foram analisadas quanto à atividade de transglutaminase. (2) Foi testada a precipitação da transglutaminase do sobrenadante do meio de cultivo com solventes 70% de acetona ou 70% de etanol resfriados a -15ºC (proporção 1:2; v:v). As misturas foram centrifugadas a 10.000 x *g* por 20 minutos a 4ºC. Após a secagem as preparações brutas foram armazenadas a -18°C. A atividade de

transglutaminase foi determinada como descrito anteriormente. (3) A liofilização de alíquotas do sobrenadante da fermentação também foi testada como um método mais direto de concentração da enzima.

Resultados

Os primeiros testes de concentração e semi-purificação da transglutaminase foram feitos através da precipitação fracionada por sulfato de amônio. O objetivo desse teste inicial era de verificar se a atividade de transglutaminase era mais expressiva em uma das frações protéicas precipitadas por uma determinada concentração de sulfato de amônio. A Tabela I.1, ilustra a atividade enzimática média (resultado de três réplicas dos testes) para o sobrenadante inicial da fermentação (sem adição de sulfato de amônio), cada uma das frações precipitadas, e para o sobrenadante da proteína precipitada a 80% de concentração do sal.

Tabela I.1. Fracionamento da transglutaminase do sobrenadante do meio de cultura com sulfato de amônio

Amostra	Atividade de MTGase (U/mL)
Sobrenadante da fermentação	0,96[a]
Precipitado com 40% de sulfato de amônio	0,38[b]
Precipitado com 60% de sulfato de amônio	1,09[a]
Precipitado com 80% de sulfato de amônio	0,69[ab]
Sobrenadante final (atividade residual)	0,027[c]

*As letras diferentes representam médias significativamente diferentes pelo teste de Tuckey, aplicado com 95% de confiança.

A Tabela I.1, mostra que todas as frações protéicas precipitadas apresentaram atividade de transglutaminase. Dessa forma, a precipitação fracionada das proteínas do

sobrenadante da fermentação não se mostrou um bom método de semi-purificação ou concentração da enzima.

O próximo teste foi comparativo entre diferentes métodos de concentração. Sendo assim, a concentração de proteína de cada amostra foi medida pelo método de Bradford (Bradford, 1976), e esse parâmetro foi mantido constante em todas as soluções das preparações enzimáticas para a determinação da atividade de MTGase em cada amostra. Para o preparo da primeira amostra, o sobrenadante da fermentação foi precipitado diretamente com 80% de sulfato de amônio e teve atividade de MTGase medida antes e depois do processo de diálise. A segunda amostra foi preparada pela precipitação das proteínas com acetona; a terceira amostra é resultado da precipitação das proteínas com etanol; e a quarta amostra foi preparada pelo congelamento e liofilização do sobrenadante da fermentação. Todos os testes foram realizados em triplicatas. Os resultados de atividade enzimática de cada amostra estão ilustrados na Tabela I.2.

Tabela I.2. Comparação entre métodos de concentração da transglutaminase

Amostra	Atividade de MTGase (U/mL)
80% de sulfato de amônio antes da diálise	0,62
80% de sulfato de amônio após a diálise	0,41
Acetona	0,27
Etanol	0,18
Liofilização	0,69

A conclusão desse estudo foi que a precipitação com sulfato de amônio (80%) leva a uma perda de atividade enzimática menor do que a utilização de solventes como

etanol e acetona para a precipitação. Porém, o processo de diálise para a retirada do sal acarreta em perda da atividade de MTGase, enquanto que o processo de liofilização do sobrenadante da fermentação se mostrou o processo mais capaz de reter a maior atividade de MTGase dentre os testados. O extrato bruto liofilizado foi congelado a -18°C, sendo que foi verificado que não houve perda significativa da MTGase após dois meses.

Referências

BRADFORD, M. M. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry. (72):** 48-254, **1976**

ANEXO III

Testes de purificação da MTGase

O início do estudo da purificação da MTgase produzida seguiu um caminho mais convencional do que a utilização de colunas de filtração em gel, como foi descrito no capítulo 3. Na primeira fase, foram testadas diferentes colunas de troca iônica, com objetivo de selecionar a resina mais adequada para a purificação da enzima.

A preparação bruta de transglutaminase liofilizada foi solubilizada em tampão, sendo que foram testadas várias concentrações para a solução de injeção. As colunas utilizadas eram de 1 mL de capacidade, e estavam conectadas a um sistema FPLC. As colunas eram parte do kit de colunas de troca iônica da Pharmacia (HiTrap IEX Selection Kit): SP Sepharose Fast Flow, SP Sepharose XL, CM Sepharose Fast Flow, Q Sepharose Fast Flow, Q Sepharose XL, DEAE Sepharose Fast Flow e ANX Sepharose 4 Fast Flow. Todas elas foram previamente equilibradas com os tampões: tampão acetato de sódio 50mM pH 4,0 para as colunas de troca de cátion, e tampão fosfato 50mM pH 6,5 para testar as colunas de troca de ânions. As colunas foram lavadas com o mesmo tampão e as frações ativas eluídas com gradiente NaCl em tampão de 0 a 1M.

No entanto, após os testes, só foi possível detectar atividade de transglutaminase nas frações protéicas recolhidas antes da eluição com gradiente salino, o que significa que as colunas não eram capazes de reter a enzima. As colunas de troca catiônica, ao contrário das de troca aniônica, apresentavam um pequeno pico de proteína durante a eluição do gradiente salino, mas sem atividade enzimática detectável. Dessa forma, uma

coluna de maior capacidade foi testada, a fim de verificar a existência de transglutaminase nessa fração protéica.

Uma coluna de CM-celulose, de capacidade igual a 60 mL, foi testada, variando-se a quantidade de enzima na injeção, o fluxo de tampão pela coluna, e o volume das frações recolhidas, de forma garantir que a diluição da enzima nas frações fosse detectável pelo método analítico de atividade de MTGase. Porém, a atividade de MTGase continuou sendo detectada apenas nas frações protéicas anteriores ao gradiente salino.

A aparente falta de interação entre a enzima e as colunas de troca iônica levaram aos testes com colunas de filtração em gel. A cromatografia em coluna Sephadex G-75 mostrou-se um método viável de purificação, como descrito no capítulo 3. A literatura reporta, com freqüência, a utilização de colunas de troca iônica como uma primeira etapa de purificação de MTGases (capítulo 1, revisão de trabalhos de purificação de transglutaminase). No entanto, etapas de pré-purificação, como precipitação fracionada com sulfato de amônio, ou filtração em membranas, estão normalmente presentes nestes trabalhos, antes das etapas de cromatografia. Uma hipótese que explicaria essa falta de interação da enzima com as colunas de troca iônica pode ser a composição do extrato bruto aplicado nas colunas. Nenhum dos métodos de pré-purificação testados no sobrenadante da fermentação pode ser utilizado, porque causavam uma perda muito acentuada da atividade enzimática do extrato. Optou-se por apenas concentrar a enzima por meio de liofilização, mas a carga de impurezas do meio de cultura semi-complexo é bastante grande, e pode ser responsável por prejudicar a interação dos grupos presentes na enzima com os grupos reativos das colunas. Nesse caso, o método de separação físico, das colunas de filtração em gel, acabou se mostrando mais adequado.