Universidade Estadual de Londrina

MICHELE ROSSET

HIDRÓLISE ENZIMÁTICA DE CARBOIDRATOS DE SOJA [*GLYCINE MAX* (L.) MERRILL] E EFEITOS EM TOFU TIPO SILKEN

Londrina
2011

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HIDRÓLISE ENZIMÁTICA DE CARBOIDRATOS DE SOJA [*GLYCINE MAX* (L.) MERRILL] E EFEITOS EM TOFU TIPO SILKEN

Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, nível Doutorado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Ciência de Alimentos.

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Dedicatória

A Deus, pela Presença e Luz;
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Toda vez que acreditares que
as tuas preces não foram ouvidas
porque não foram atendidas,
pensa que tudo está certo.
Logo mais ou um pouco depois
descobrirás que Deus estava certo
em Se manter silente.
Tenha certeza: nada te acontece que
não seja o melhor para ti, naquele momento.
***Isso porque Deus nunca Se engana**.*

Anônimo

Conclusão de uma mensagem baseada
em uma lenda norueguesa

ROSSET, Michele. **Hidrólise Enzimática de Carboidratos de Soja [*Glycine Max* (L.) Merrill] e Efeitos em Tofu tipo Silken.** 2011. 102 f. Tese (Doutorado em Ciência de Alimentos) – Universidade Estadual de Londrina, Londrina, 2011.

RESUMO

A adição de Viscozyme L na suspensão de soja pode resultar em um tofu com características diferentes do tradicional. Devido à ação da enzima, material da parede celular (polissacarídeos) poderá ser parcialmente hidrolisado liberando mono e oligossacarídeos, os quais serão transferidos para o tofu, provavelmente modificando sua composição e textura. O objetivo deste trabalho foi estudar os efeitos, em tofu tipo silken, da hidrólise de carboidratos de soja realizada pelo complexo enzimático Viscozyme L. Na primeira etapa, foi realizada a otimização da temperatura de ação de Viscozyme L e foi verificado que, a 55 oC, os teores dos açúcares redutores aumentaram em até 4 vezes, comparado ao controle. Estaquiose foi o oligossacarídeo predominante no tofu tratado (4,58 g/100 g) e o conteúdo de rafinose foi de 1,22 e 0,75 g/100 g nos tofus tratado e controle, respectivamente. O nível de glicose, aproximadamente, duplicou no tofu tratado (1,66 g/100 g) em relação ao controle (0,74 g/100 g). O tofu tratado apresentou maior quantidade de compostos fenólicos que o controle (173 e 161 mg equivalentes de ácido gálico/100 g de tofu liofilizado, respectivamente) e maior atividade antioxidante pelo teste ABTS e DPPH. O conteúdo total de isoflavonas (92 mMol/100 g tofu) não apresentou diferença entre as amostras, mas o tofu tratado apresentou maior concentração de malonil glicosídeos e o controle de β-glicosídeos. Os tofus apresentaram diferenças sensoriais como maior odor de soja e menor uniformidade da superfície (tofu tratado), mas não houve preferência de uma amostra em relação à outra. O tofu tratado teve maior quantidade de glicose e frutose que o controle, porém não foram verificadas diferenças nos gostos (ácido e amargo) das amostras. Isto pode ter ocorrido pelo fato dos tofus terem sido coagulados com glucona-delta-lactona, um coagulante ácido que pode ter mascarado o sabor doce do tofu tratado com enzima. As condições ideais de temperatura e concentração de Viscozyme L para extração de proteína foram 60 oC e 30 FBG (Fungal Beta Glucanase) por 30 minutos. O pré-tratamento enzimático para extração de proteínas resultou em rendimento de 56,27%, superior ao método alcalino tradicional, 33,04%; o efeito da temperatura de pré-tratamento foi a variável mais importante. Para hidrólise de carboidratos, as condições ótima de temperatura e concentração de enzima foram 45 oC e 45 FBG/10 g de farinha desengordurada de soja, respectivamente. Ambas amostras de tofu apresentaram aglomerados de microestruturas globulares de proteínas e estrutura tridimensional fibrosa, típica de tofus.

Palavras – chave: Proteínas. Carboidratos. Antioxidantes. Isoflavonas. Derivados de soja. Viscozyme L.

ROSSET, Michele. **Enzymatic Hydrolisis of Soy [*Glycine Max* (L.) Merrill] Carbohydrates and Effects on Silken Tofu.** 2011. 102 f. Thesis (Doctorate in Food Science) – Universidade Estadual de Londrina, Londrina, 2011.

ABSTRACT

The addition of Viscozyme L in soy suspension may result in a tofu with characteristics differents from traditional. Due to the presence of the enzyme, cell wall material (polysaccharides) may be partially hydrolyzed to release mono-and oligosaccharides, which will be transferred to the tofu and probably influencing composition and texture. The aim of this work was to study the effects, in silken tofu, of the hydrolysis of soy carbohydrates by the enzyme complex of Viscozyme L. First, this study investigated the enzymatic pre-treatment of soy slurry to optimize conditions of the enzyme action. The optimum temperature of Viscozyme L was 55 oC and it was found that the levels of reducing sugars increased up to 4 times compared to the control. Stachyose was the predominant oligosaccharide in treated tofu 4.58 g/100 g, and of raffinose was 1.22 and 0.75 g/100 g in treated tofu and control, respectively. The glucose level was approximately doubled in the treated tofu (1.66 g/100 g) compared to control (0.74 g/100 g). The treated tofu had a higher amount of phenolic compounds compared to the control, 173 and 161 mg of gallic acid equivalent/100 g of dried tofu, and higher antioxidant activity by ABTS and DPPH test. The total content of isoflavonas (92 mMol/100 g tofu) did not differ between the samples but the treated tofu had a higher concentration of malonyl glycosides and the control of β-glycosides. The tofus showed sensory differences as the largest soybean odor and less uniform surface (treated tofu), but there was no preference for one sample over the other. The treated tofu had higher amount of glucose and fructose than the control, but there was no observed differences in the taste (acid and bitter) of the samples. This may have occurred because the tofus were coagulated with glucona-delta-lactone, an acid coagulant that may have masked the sweet taste of treated tofu. The optimum conditions of temperature and concentration of Viscozyme L for protein extraction were 60 ° C and 30 FBG (Fungal Beta glucanase), during 30 minutes. Enzimatic pre-treatment for proteins extraction resulted in a yield of 56.27%, higher than the traditional alkaline method, 33.04%; the effect of the pre-treatment temperature was the most important variable. For carbohydrate hydrolysis the optimal conditions of temperature and enzyme concentration were 45° C and 45 g of FBG/10 g defatted soy flour. All tofu samples had a globular microstructure of protein which was integrated into clumps and showed a fibrous three-dimensional network structure, typical of tofu.

Keywords: Proteins. Carbohydrates. Antioxidants. Isoflavones. Soy products. Viscozyme L.

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2‘-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ANOVA	Analysis of Variance
ANVISA	Agência Nacional de Vigilância Sanitária
AF	Ácido Fítico
AOAC	Association of Official Analitycal Chemists
CONAB	Companhia Nacional de Abastecimento
DPPH	1,1-diphenyl-2-picryhydrazyl
EGU	Endo-Glucanase Units
EHS	Extrato Hidrossolúvel de Soja
FBG	Fungal Beta Glucanase
FDS	Farinha Desengordurada de Soja
FRAP	Ferric Reducing Antioxidant Power
GAE	Equivalentes de Ácido Gálico
GDL	Glucona-Delta-Lactona
GluE	Equivalente de Glicose
h	Hora
HPLC	High Performance Liquid Chromatography
M	Molar
min	Minuto
NC	Novo Celulase
PNCs	Polissacarídeos não Celulósicos
Rpm	Rotação por minuto
s	Segundos
TE	Equivalentes de Trolox
TPTZ	2,4,6-tripyridyl-s-triazine
WHC	Water Holding Capacity

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1 INTRODUÇÃO

Estudos mostrando a relação entre dieta e saúde, somados ao crescente interesse de pessoas em consumir alimentos mais saudáveis, têm levado a indústria alimentícia ao desenvolvimento de novos produtos, cujas funções pretendem ir além do fornecimento de nutrientes básicos e da satisfação do paladar do consumidor. Esses produtos são conhecidos como alimentos funcionais e têm como principal função a redução do risco de doenças crônico-degenerativas. Eles representam um novo segmento dentro do mercado de alimentos e possuem como principais apelos de venda suas alegações de saúde, que são veiculadas pelo rótulo e pela propaganda. No Brasil, o mercado de alimentos funcionais é ainda incipiente, porém com um grande potencial, considerando-se a disponibilidade de fontes naturais, a capacidade produtiva da indústria local e o tamanho do mercado consumidor (BEHRENS & DA SILVA, 2004).

Dentre os alimentos cujas alegações de saúde têm sido amplamente divulgadas pela mídia nos últimos anos destaca-se a soja. A inclusão da leguminosa e seus derivados como parte da dieta diária é recomendável, pois contribuem para prover os nutrientes necessários para o desenvolvimento, crescimento e manutenção do organismo, além de fornecer componentes, tais como antioxidantes naturais, isoflavonas e fosfolipídeos, entre outros, que auxiliam no bem-estar físico, melhorando o funcionamento do organismo e prevenindo doenças crônico-degenerativas (MORAES, HAJ-ISA, ALMEIDA, MORETTI, 2006).

Dentro da versatilidade da soja no campo da indústria de alimentos, são conhecidos e comercializados, além da soja em grãos, farinha de soja, concentrados e isolados de soja, soja texturizada, alimentos fermentados como missô, shoyo, tempeh, e ainda, o extrato hidrossolúvel de soja (EHS) e tofu.

O tofu é o produto obtido da precipitação das proteínas, pela adição de coagulante, dentre os utilizados tem-se a glucona-delta-lactona (GDL), sais de cálcio e magnésio (sulfato de cálcio, sulfato de magnésio, cloreto de cálcio e cloreto de magnésio), produzindo um gel resultante da formação de uma rede protéica com retenção de água, lipídeos e outros constituintes. Sua textura é uniforme, macia e elástica, sendo importante fonte de proteína, minerais e vitaminas, ao mesmo tempo em que apresenta baixa proporção de gorduras saturadas e ausência total de

colesterol. Como alimento saudável, de alto valor nutritivo e de custo reduzido, o tofu tem sido utilizado em preparações alimentícias, em substituição de ovos, queijos, carnes e outros alimentos de origem animal (LIU, 1997; CIABOTTI, BARCELLOS, PINHEIRO, CLEMENTE & LIMA, 2006).

Os carboidratos, principalmente oligossacarídeos, têm sido considerados componentes importantes (prebióticos) na manutenção e multiplicação de microrganismos probióticos no intestino humano. Esta fração tem sido estudada ou associada a fatores benéficos à saúde em produtos integrais de soja, mas não nos produtos derivados. Tratamentos para hidrólise com hidrolases são comuns para proteínas, mas pouco estudado para hidrólise de carboidratos em soja.

Além disso, carboidrases têm sido empregadas diretamente para promover a maceração ou desintegração da parede celular do alimento facilitando, subseqüentemente, a extração do nitrogênio (ANSHARULLAH & COLIN, 1997; TANG, HETTIARACHCHY, ESWARANANDAM & CRANDALL, 2003; GUAN & YAO, 2008).

A adição do complexo multienzimático Viscozyme L na suspensão de soja, pode resultar em um tofu com características diferentes do tradicional. Devido a ação da enzima, material da parede celular (polissacarídeos) poderá ser parcialmente hidrolisado liberando mono e oligossacarídeos, os quais serão transferidos para o tofu, provavelmente modificando a composição e textura. O objetivo deste trabalho foi estudar os efeitos, em tofu tipo silken, da hidrólise de carboidratos de soja realizada pelo complexo enzimático Viscozyme L.

2 REVISÃO BIBLIOGRÁFICA

2.1 SOJA

2.1.1 Breve Histórico, Características e Situação Atual da Soja

Botanicamente, a soja pertence à família *Leguminosae,* à subfamília *Papilionoideae* e ao gênero *Glycine,* L. A variedade mais cultivada é denominada *Glycine max* (L.) Merril. Acredita-se que a soja originou-se na China, provavelmente nas regiões norte e central, há cerca de 4000 a 5000 anos e foi registrada pelo imperador chinês Shen Nong, por volta de 2838 a.C. (LIU, 1997).

Nos Estados Unidos, a soja passou a ser utilizada a partir de 1804. Primeiramente, o processamento visava apenas a obtenção de óleo, com toda a massa restante destinada à alimentação de gado leiteiro e ao uso como fertilizante. Após a Segunda Guerra Mundial, quando foi detectada uma deficiência mundial de proteínas para alimentação humana, foi dada atenção especial à soja havendo grandes avanços em novas tecnologias para viabilização e aceitação de suas proteínas na alimentação humana (LIU, 1997).

No Brasil, a soja foi efetivamente introduzida em 1914 no Estado do Rio Grande do Sul, porém o verdadeiro impulso na produção da soja no Brasil ocorreu somente na década de 60, quando se iniciou o cultivo sucessivo trigo-soja no Rio Grande do Sul. No final dessa década e na seguinte, começaram a ser desenvolvidas, por melhoramento genético, as primeiras cultivares brasileiras de alto rendimento e adaptadas às nossas condições climáticas (MORAIS & SILVA, 1996).

A produção mundial de soja para a safra 2010/2011 foi estimada em 263,7 milhões de toneladas (EMBRAPA, 2011). De acordo com o 12º Levantamento de Grãos, realizado em Setembro de 2011 pela Companhia Nacional de Abastecimento (CONAB), a produção brasileira estimada em 75,32 milhões de toneladas manteve o ritmo de crescimento das últimas safras. Este volume é 9,7% ou 6,64 milhões de toneladas superior à produção obtida na safra 2009/10, quando foram colhidas 68,69 milhões de toneladas (CONAB, 2011).

2.1.2 Componentes da Soja

A composição da semente de soja depende de fatores como variedade, época de plantio, local geográfico e clima. Em média, a soja é constituída de 40% de proteína, 35% de carboidratos, 20% de lipídeos e 5% de cinzas (Tabela 1). A soja contém muitas substâncias secundárias como fitatos, oligossacarídeos e isoflavonas (LIU, 1997).

Tabela 1 – Composição centesimal do grão de soja e de suas partes estruturais.

Parte	Proteínas	Lipídeos	Carboidratos	Cinzas
Grão inteiro	40	20	35	5
Cotilédone	43	23	29	5
Casca	9	1	86	4,3
Hipocótilo	41	11	43	4,4

Fonte: adaptado de LIU (1997).

Dentre os componentes funcionais da soja destacam-se as proteínas e isoflavonas. Os grãos de soja ainda contêm carboidratos, com teor mais elevado na casca, e oligossacarídeos (estaquiose e rafinose) que são hidrolisados pelas enzimas invertase e α-galactosidase. Esses oligossacarídeos são reduzidos com a maceração e o cozimento dos grãos (LIU, 1997) e na fabricação de tofu prensado (soft), são eliminados no soro (VAN DER RIET, WIGHT, CILLIERS & DATEL, 1989).

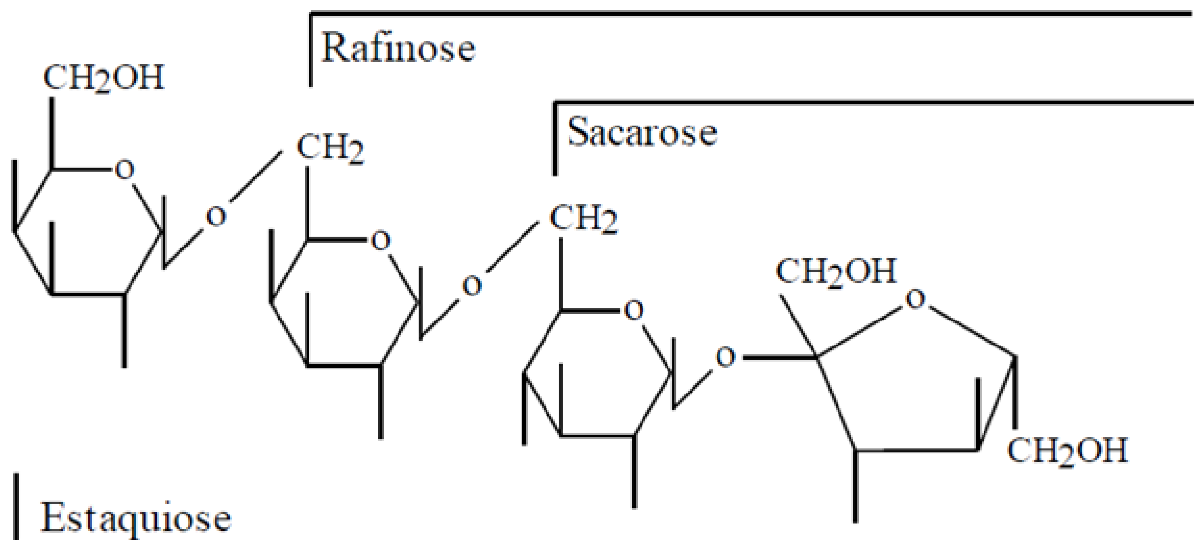
A quantidade de isoflavona presente no grão de soja é influenciada por uma série de fatores genéticos e ambientais, safra e localização. Temperatura do meio ambiente durante o desenvolvimento das sementes é um fator determinante para o conteúdo das isoflavonas nas sementes de soja, sendo que altas temperaturas diminuem essas quantidades (CARRÃO-PANIZZI, SIMÃO & KIKUCHI, 2003). Existem relatos de uma maior razão de malonil-glicosídeos em grãos de soja, entretanto as agliconas são encontradas em maior quantidade em produtos fermentados (WANG & MURPHY, 1994a).

2.1.2.1 Carboidratos

Embora os carboidratos sejam o segundo maior grupo de componentes na soja (Tabela 1), poucos estudos são encontrados na literatura sobre este grupo devido sua menor importância econômica comparada ao óleo (LIU, 1997).

Mais de 99% dos açúcares presentes em grãos de soja maturos são sacarose e oligossacarídeos como rafinose e estaquisose (HYMOWITZ, WALKER, COLLINS & PANCZNER, 1972a). A sacarose se encontra na faixa de 2,5 – 8,2%, rafinose entre 0,1 – 0,9% e estaquiose entre 1,4 – 4,1%, em base seca, variando em diferentes cultivares e condições de cultivo (HYMOWITZ, WALKER, COLLINS & PANCZNER, 1972b). Basicamente, os oligossacarídeos em soja são açúcares não redutores, contendo unidades de frutose, glicose e galactose (Figura 1).

Figura 1 – Estrutura de oligossacarídeos encontrados em soja.



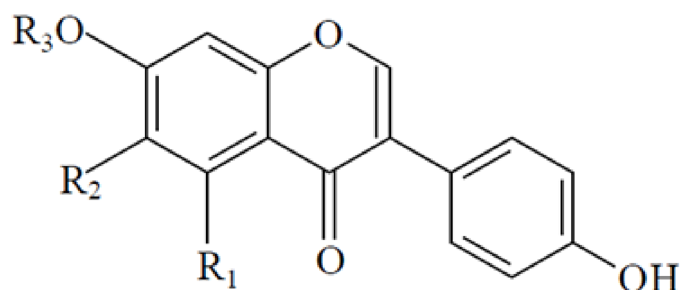
Entre os açúcares solúveis, rafinose e estaquiose recebem mais atenção pois a presença destes está relacionada com flatulência e desconforto abdominal associado ao consumo de derivados de soja. Quando consumidos, esses oligossacarídeos não são digeríveis no intestino devido a ausência da enzima α-galactosidade, responsável pela hidrólise desses açúcares. Isto resulta na produção de gases como dióxido de carbono, hidrogênio e nitrogênio (LIU, 1997).

No entanto, estudos têm mostrado efeitos benéficos desses oligossacarídeos como aumento da população de bifidobactéria no cólon, prevenção de constipação devido à produção de ácidos graxos de cadeia curta pelas bifidobactérias, redução da pressão sanguínea, efeitos anti-câncer e produção de nutrientes como vitaminas, também relacionada ao aumento da atividade de bifidobactéria (TOMOMATSU, 1994; MUSSATTO & MANCILHA, 2007; QIANG, YONGLIE & QIANBING, 2009) ou seja, tornam-se componentes efetivos de alimentos funcionais.

2.1.2.2 Isoflavonas

As isoflavonas são consideradas uma classe de fitoestrógeno que estão presentes na soja e têm demonstrado atividade biológica. O grão de soja contém basicamente três tipos de isoflavonas que se apresentam normalmente em quatro diferentes formas, glicosiladas (daidzina, genistina e glicitina); formas acetilglicosiladas (acetildaidzina, acetilgenistina e acetilglicitina); formas malonilglicosiladas (malonildaidzina, malonilgenistina e malonilglicitina) e na forma estrutural não conjugada, aglicona (daidzeína, genisteína e gliciteína) (KUDOU, FLEURY, WELTI, MAGNOLATO, UCHIDA, KITAMURA & OKUBO, 1991; WANG & MURPHY, 1994a). A estrutura geral das isoflavonas pode ser observada na Figura 2.

Figura 2 – Estrutura química de 12 isoflavonas isoladas de soja.



Nome	R_1	R_2	R_3
Daidzeína	H	H	H
Gliciteína	H	OCH_3	H
Genisteína	OH	H	H
Daidzina	H	H	Glu
Glicitina	H	OCH_3	Glu
Genistina	OH	H	Glu
Acetildaidzina	H	H	Glu-$COCH_3$
Acetilglicitina	H	OCH_3	Glu-$COCH_3$
Acetilgenistina	OH	H	Glu-$COCH_3$
Malonildaidzina	H	H	Glu-$COCH_2COOH$
Malonilglicitina	H	OCH_3	Glu-$COCH_2COOH$
Malonilgenistina	OH	H	Glu-$COCH_2COOH$

A forma malonil tem sido descrita como a principal isoflavona constituinte das sementes de soja, embora ela seja termicamente instável e convertida em sua correspondente isoflavona glicosídica (KUDOU *et al*., 1991).

Em soja crua as três formas de agliconas genisteína, daidzeína e gliciteína são encontradas na razão de aproximadamente 6:3:1, respectivamente. A concentração de isoflavonas em soja não processada em média é de 1,2-4,2 mg/100 g, com grande variação devido à variedade, ano de colheita e localização do plantio (WANG & MURPHY, 1994b).

No homem, as agliconas são absorvidas mais rapidamente e em maiores quantidades que seus glicosídeos (IZUMI, PIKULA, OSAWA, OBATA, TOBE, SAITO, KATAOKA, KUBOTA & KIKUCHI, 2000) pois apresentam menor

massa molar e baixa hidrofobicidade. Estudos têm demonstrado que as funções fisiológicas variam entre as isoflavonas. Por exemplo, em relação à ação anticancerígena, a genisteína tem sido estudada principalmente por inibir o crescimento de células do câncer de próstata (PETERSON & BARNES, 1993). Mulheres japonesas que possuem uma dieta rica em isoflavonas apresentam menores incidências de câncer de mama (FUKUTAKE, TAKAHASHI, ISHIDA, KAWAMURAI, SUGIMURA & WAKABAYASHI, 1996). Estudos em animais também mostraram que a adição de soja ou isoflavonas em suas dietas reduziram entre 25 a 50% o número de células cancerígenas (BARNES, GRUBBS, SETCHELL & CARLSON, 1990; LAMARTINIERE, ZHAO & FRITZ, 2000; BEDANI & ROSSI, 2005).

Fukutake *et al.* (1996) quantificaram genistina e genisteína em soja e produtos derivados. Os teores de genisteína e genistina em grãos de soja e farinha de soja foram de 4,6 a 18,2 e 200,6 to 968,1 µg/g, respectivamente. Os teores encontrados pelos autores para extrato hidrossolúvel de soja e tofu foram de 1,9 a 13,9 e 94,8 a 137,7 µg/g, respectivamente. Em produtos fermentados como missô e natto foram quantificados 38,5 a 229,1 µg/g para genisteína e 71,7 a 492,8 µg/g para genistina. É possível observar que os níveis de genisteína em produtos fermentados foram superiores aos encontrados nos grãos, extrato de soja e tofu. A partir desses resultados, os autores concluíram que a ligação β-glicosídica presente na molécula de genistina é quebrada por microrganismos durante a fermentação gerando genisteína.

Hui, Henning, Park, Heber e Go (2001) determinaram a variação de isoflavonas em 23 diferentes marcas comerciais de tofus. O conteúdo de genistina e daidzina/glicitina varia de 0,07 a 0,34 mg/g e 0,10 a 0,24 mg/g (base úmida), respectivamente.

Os autores também verificaram uma variação de 28% entre lotes dentro de uma mesma marca.

As condições de processamento da soja podem causar mudanças no conteúdo e no perfil das isoflavonas. Maceração, processamento térmico e coagulação em produtos processados de soja têm sido reportados por aumentar o conteúdo de isoflavonas (WANG & MURPHY, 1996), porém são observadas importantes perdas de isoflavonas, entre outros compostos, como é o caso do processamento do tofu, onde parte das isoflavonas é eliminada no soro. A

estocagem é também conhecida por alterar o perfil de isoflavonas em produtos de soja (KIM, KIM, HAHN & CHUNG, 2005).

Kao, Lu, Hsieh e Chen (2004) estudando a estabilidade de isoflavonas glicosídicas durante o processamento do extrato de soja e tofu observaram uma maior formação de genisteína durante a maceração, seguida de daidzeína e gliciteína. O conteúdo total de isoflavonas pode ser alterado com o emprego de altas temperaturas através da conversão das derivadas. Das quatro formas de isoflavonas, somente as agliconas mostraram uma tendência ao aumento durante a maceração com o tempo e temperatura. Isto é devido à hidrólise dos glicosídeos realizada pela β-glicosidase em soja durante a maceração (WANG & MURPHY, 1996). Verificou-se um maior rendimento de agliconas durante a maceração a 45 °C do que 25 e 35 °C, podendo ser devido à enzima β-glicosidase possuir maior atividade, próxima de 50 °C, e os níveis de agliconas aumentarem ao máximo nesta temperatura pela hidrólise dos glicosídeos.

Pesquisas têm revelado que o processo de coagulação pode ser responsável por uma perda considerável no teor de isoflavonas em tofus (WANG & MURPHY, 1996; LIU, 1997; JACKSON, DINI, LAVANDIER, RUPASINGHE, FAULKNER, POYSA, BUZZELL & DEGRANDIS, 2002). Jackson *et al*. (2002) observaram que as isoflavonas podem formar complexos com as proteínas, os quais podem ser liberados junto com o soro. Deste modo, quanto maior a concentração de coagulante, maior a velocidade do processo de coagulação, e com isso maior o processo de sinérese (SUN & BREENE, 1991), resultando na perda das isoflavonas.

Kao *et al.* (2004) verificaram que tofu feito com menores concentrações de coagulante (0,3% de sulfato de cálcio) apresentou maior rendimento no teor de isoflavonas comparado as demais concentrações utilizadas no estudo (0,5% e 0,7%). Comparando as concentrações mais elevadas, utilizando sulfato de cálcio e cloreto de cálcio na mesma concentração, o maior rendimento de isoflavonas foi obtido com sulfato de cálcio. Provavelmente pelo fato deste sal ser melhor coagulante de proteínas comparado ao cloreto.

Jackson *et al*. (2002) verificaram que o teor médio de isoflavonas recuperados no extrato hidrossolúvel de soja e no tofu em relação à concentração inicial presente nos grãos de soja foram de 54 e 36%, respectivamente. A porcentagem estimada da perda total de isoflavonas na água utilizada para macerar

os grãos, no okara e no soro foi de 4, 31, e 18%, respectivamente. Durante o processamento, os níveis detectáveis de agliconas, grupos glicosídeos e acetilglicosídeos aumentaram, enquanto que os grupos malonilglicosídeos diminuíram.

Em produtos à base de soja a distribuição dos compostos de isoflavonas depende das condições de processamento, principalmente da temperatura de tratamento do material (WANG & MURPHY, 1996). As formas malonilglicosídicas são termicamente instáveis, sofrendo hidrólise pela ação de temperaturas acima de 80 ºC (KUDOU *et al.*, 1991), dando origem às formas β-glicosídicas (BARNES, KIRK & COWARD, 1994). Segundo Barnes et al. (1994), os malonilglicosídeos pela ação de calor, podem originar os β-glicosídeos e acetilglicosídeos.

Coward, Smith, Kirk e Barnes (1998) analisaram a presença dos 12 compostos de isoflavonas, e encontraram 130 mg de isoflavonas totais/100 g de farinha de soja, sendo o composto em maior concentração os malonídeos. Coward, Barnes, Setchell e Barnes (1993), ao analisarem produtos à base de soja concluíram que processos de aquecimento promovem descarboxilação de conjugados malonil originando conjugados acetil-glicosídios, pois estes são detectados apenas em produtos submetidos a tratamento térmico durante a produção.

Góes-Favoni, Beléia, Carrão-Panizzi e Mandarino (2004) determinaram o teor de isoflavonas em produtos comerciais à base de soja produzidos no Brasil. Em farinha de soja e proteína texturizada predominaram os compostos malonil-conjugados; a farinha de soja (96 mg de agliconas equivalentes/100g) e proteína texturizada (70 mg de agliconas equivalentes/100g), obtidas a partir da mesma cultivar apresentaram diferenças na concentração e distribuição dos isômeros devido ao processamento. Extratos hidrossolúveis e formulados infantis apresentaram agliconas, variando de 8% a 28% do total de isoflavonas, mas os principais isômeros foram os β-glicosídeos. Em formulados infantis a concentração de isoflavonas totais foi menor que nos demais produtos devido à adição de ingredientes não derivados de soja. Os autores concluíram que os tratamentos térmicos elevaram as concentrações de β-glicosídeos e agliconas, enquanto compostos malonil tiveram a concentração reduzida.

O clima, localização e cultivar influenciam nos teores de isoflavonas e seus derivados glicosídicos na soja (ELDRIDGE & KWOLEK, 1983; WANG & PURPHY, 1994b; CARRÃO-PANIZZI & BORDINGNON, 2000; CARRÃO-PANIZZI, BERHOW, MANDARINO & OLIVEIRA, 2009). Eldridge e Kwolek (1983) estudaram os efeitos da variedade e condições ambientais nos níveis de isoflavonas em soja. O total de isoflavonas variou de 116 a 309 mg/g com o tipo de cultivar e de 46 a 195 mg/g na mesma cultivar, porém cultivada em localidades diferentes. Além disso, houve variação nos teores quando plantadas no mesmo local, porém em safras diferentes.

Carrão-Panizzi, Berhow, Mandarino e Oliveira (2009) avaliaram as concentrações de isoflavonas nas sementes de diferentes cultivares brasileiras de soja. Sementes de 233 cultivares semeadas em Ponta Grossa, PR, na safra de 2001/2002, e sementes de 22 cultivares plantadas em diferentes locais das regiões Nordeste, Sudeste e Sul foram analisadas quanto ao teor total de isoflavonas, incluindo daidzina, glicitina, genistina e acetilgenistina. O total de isoflavonas variou entre 12 mg /100 g (cv. Embrapa 48) e 461 mg /100 g (cv. CS 305) entre as 233 cultivares plantadas em Ponta Grossa, e essas diferenças foram devidas aos efeitos genéticos, pois todas foram cultivadas e colhidas no mesmo local e ano. Isso indica que há possibilidade de melhoramento genético para o teor de isoflavonas. Diferenças no teor de isoflavonas observadas nas cultivares plantadas nos diferentes locais de semeadura permitem selecionar locais segundo a concentração ótima de isoflavonas (reduzida ou alta), dependendo do uso da soja.

2.2 A Soja e Seus Derivados

O alto teor de sacarose presente na soja (2,5 – 8,2%) (HYMOWITZ *et al*, 1972b) é desejável, pois é um componente responsável pelo sabor em alimentos a base de soja. Os oligossacarídeos rafinose e estaquiose, que resistem a digestão devido a presença de ligações α-galactosídicas em suas estruturas, são responsáveis por gerar flatulência e desconforto abdominal quando se consome produtos de soja. Porém, esses oligossacarídeos apresentam características prebióticas que estão relacionadas a diminuição do nível de colesterol no sangue e

pressão arterial e prevenção de alguns tipos de câncer (QIANGA, YONGLIE & QIANBING, 2009).

Dentro da versatilidade da soja [*Glycine max* (L.) Merrill] no campo da indústria de alimentos, são conhecidos e comercializados, além da soja em grãos, farinha de soja, concentrados e isolados de soja, soja texturizada, alimentos fermentados como missô, shoyo, tempeh, e ainda, o extrato hidrossolúvel e tofu, além de outros produtos.

2.2.1 Farinha Desengordurada de Soja

A maior parte da produção de soja se destina à extração de óleo, e seus resíduos são destinados, especialmente, à alimentação animal. Entretanto, a soja e seus derivados constituem matérias-primas altamente promissoras para uso na indústria de alimentos, sobretudo em produtos à base de cereais e de carnes. A adição apropriada de derivados de soja resulta em produtos alimentícios menos calóricos, com teor de lipídeos reduzido e com elevado conteúdo de proteína adequada às necessidades nutricionais de indivíduos (SILVA, NAVES, OLIVEIRA & LEITE, 2006).

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), a farinha desengordurada de soja é o produto obtido a partir dos grãos de soja convenientemente processados até a obtenção da farinha desengordurada. A remoção dos lipídeos durante o processamento resulta na concentração dos demais constituintes. O produto é utilizado como fonte de proteína para outros alimentos. Consiste em aproximadamente 50% de proteína, 40% de carboidratos, 6,5% de cinzas, 4% de fibras e 2% de lipídeos (BRASIL, 2010).

Bainy, Tosh, Corredig, Poysa e Woodrow (2008) hidrolisaram fibras de farinha desengordurada de soja de diferentes cultivares e determinaram os teores de monossacarídeos. Os resultados demonstraram diferenças no teor de açúcares solúveis entre as 12 variedades de farinhas estudadas; cinco variedades apresentaram uma média de 10,2% de açúcar enquanto que três apresentaram abaixo da média (8,5%) e quatro acima da média (11,6%).

Os teores de sacarose variaram entre 3,7 e 5,5%. O alto teor de sacarose em soja é favorável ao desenvolvimento de um sabor agradável em seus

derivados. Além disso, os açúcares solúveis são importantes para processos fermentativos, típicos da fabricação de natto e missô. Com relação aos oligossacarídeos, a quantidade presente nas diferentes farinhas variou entre 4,5 a 6,5%. Como citado anteriormente, esses açúcares são responsáveis por causarem flatulência através da ingestão de derivados de soja. Cultivares com menores teores de oligossacarídeos favorecem a produção de derivados de soja, no entanto, quantidades elevadas desses açúcares favorecem aplicações prebióticas (Bainy *et al.,* 2008). Devido à variabilidade nos teores de açúcares, diferentes aplicações são possíveis para cada cultivar.

2.2.2 Tofu

O tofu é feito a partir da coagulação do EHS aquecido; consiste no principal alimento derivado da soja presente na dieta oriental e devido ao seu sabor suave, as características de textura são as que principalmente influenciam na qualidade do tofu e na aceitabilidade pelo consumidor (KARIM, SULEBELE, AZHAR & PING, 1999). O tofu contém em média, 88% de água, 6% de proteína, 3,5% de lipídeos, 1,9% de carboidratos e 0,6% de cinzas (MIN, YU & ST MARTIN, 2005).

Existem dois métodos de preparo de EHS, nama-shiboriǁ e kanetsu-shiboriǁ. No primeiro, utilizado principalmente em países asiáticos, o EHS é processado através da filtração da suspensão seguida do aquecimento; já no segundo método, o qual é usado pelos produtores de tofu no Japão, a filtração da suspensão é feita após o aquecimento da mesma. Deste modo, as diferenças entre as propriedades dos EHS obtidos a partir destes dois métodos têm sido estudadas. O rendimento de proteínas, lipídeos e sacarídeos é influenciado pela temperatura de filtração da suspensão (TODA, CHIBA & ONO, 2007).

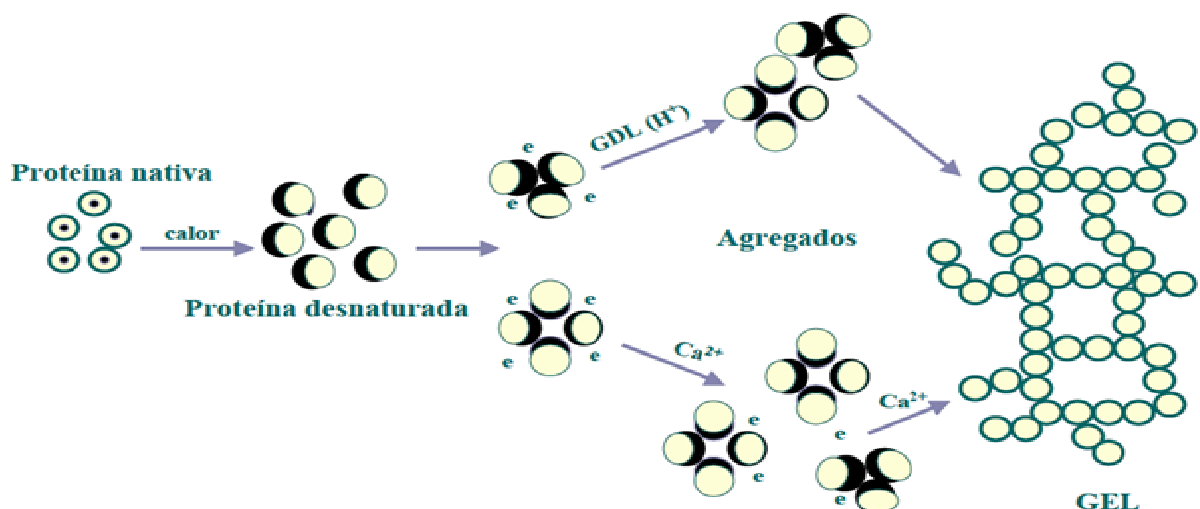
Toda *et al*. (2007) verificaram o efeito dos componentes extraídos do okara nas propriedades físico-químicas de EHS e na textura do tofu. O EHS foi preparado pelo método nama-shiboriǁ; além disso, os autores adicionaram o okara já separado em outra porção de EHS, o qual foi denominado de semi-kanetsu-shiboriǁ. Foi observado que o conteúdo de proteína precipitada foi maior no semi-kanetsu-shiboriǁ (62%) ao nama-shiboriǁ (51%), assim como a viscosidade, 7,52 e 3,56, mPa.s, respectivamente. A dureza do tofu preparado pelo método semi-kanetsu-

shibori‖ foi superior (14.6 kPa) ao nama-shibori‖ (12,8 kPa), o que pode ser devido à maior quantidade de proteína precipitada, estando de acordo com Guo e Ono (2005). O conteúdo de sólidos foi similar em ambos os métodos.

O aquecimento do EHS é importante não apenas para melhorar seu valor nutricional e reduzir o sabor característico dos produtos derivados de soja (beany flavour), mas também para que ocorra a desnaturação protéica promovendo a coagulação do EHS na presença de um coagulante (LIU, 1997).

O processo de coagulação do tofu ocorre em duas etapas (Figura 3); na primeira, a desnaturação protéica é causada pelo aquecimento do EHS e coagulação hidrofóbica promovida pelos prótons do coagulante glucona-delta-lactona ou cátions do sulfato de cálcio. Antes do aquecimento do EHS, estes grupos hidrofóbicos estão nos seus estados nativos, localizados para dentro da estrutura molecular. Com a ação do calor, estas regiões são expostas e os grupos SH (carregados negativamente), são neutralizados pelos prótons da GDL ou cátions do sulfato de cálcio (segunda etapa). Deste modo, as interações hidrofóbicas das moléculas protéicas neutralizadas tornam-se predominantes, conduzindo à coagulação. O gel é formado a partir da agregação destas moléculas e se torna denso quando ocorre próximo ao ponto isoelétrico, que é atingido com a queda do pH durante a adição do coagulante (KOHYAMA, SANO & DOI, 1995).

Figura 3 – Mecanismo de formação de gel durante o processo de coagulação das proteínas de soja na presença de GDL ou CaSO4. Círculos: moléculas de proteínas; áreas em preto: regiões hidrofóbicas.



Fonte: Kohyama *et al*. (1995).

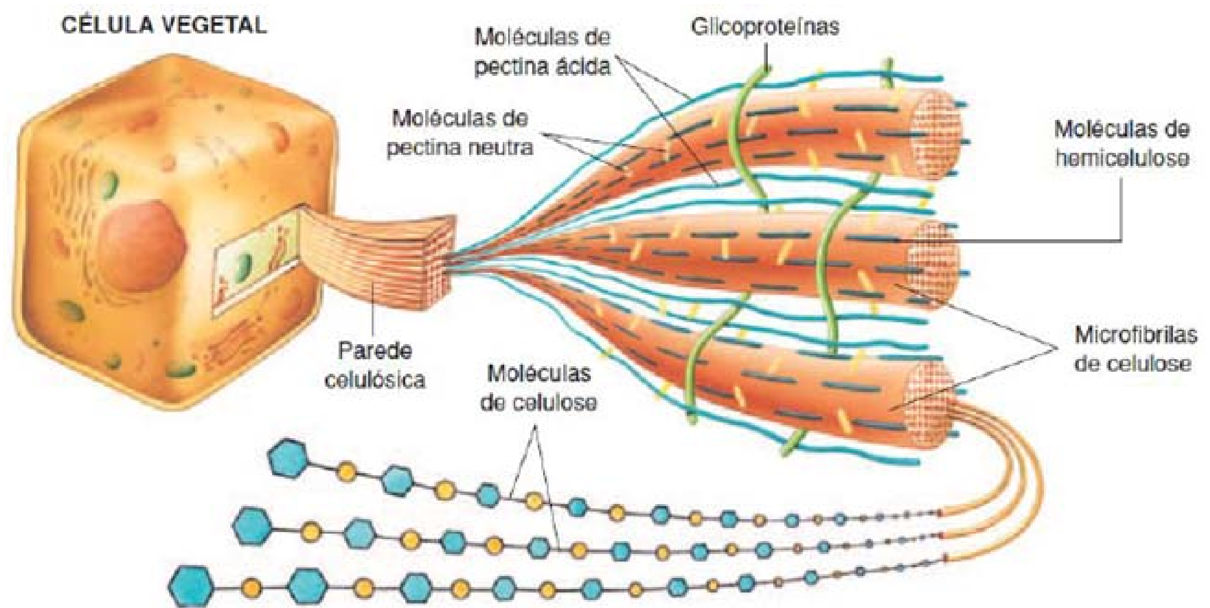
A coagulação é considerada a principal etapa no processo de produção de tofu. Nesta fase, o tipo e concentração do coagulante, temperatura do EHS na qual o coagulante deverá ser adicionado, velocidade de agitação e maneira de misturar e acrescentar este coagulante são fatores que devem ser considerados. Os coagulantes mais utilizados na produção de tofu são sulfato de cálcio, cloreto de magnésio e GDL (LIU, 1997).

A glucona-delta-lactona é o coagulante ácido mais utilizado; apresenta-se na forma de pó fino, com cor branca, inodoro e gosto adocicado. Este coagulante é preparado a partir de amido de milho, seguido de um processo fermentativo. Quando dissolvido em água, inicialmente com pH 3,6, ocorre sua hidrólise a ácido glutâmico (pH 2,5). A grande vantagem no uso de GDL é que pode ser misturado com EHS frio e então colocado num recipiente contendo água quente sob incubação. Após sua imersão, começará o processo de coagulação, resultado da hidrólise de GDL a ácido glutâmico. GDL é muito utilizado na produção de tofu tipo silken (sem remoção de soro), resultando num tofu de textura macia e fina e com rendimento alto, já que não há dessoragem. No entanto, sob o ponto de vista nutricional, os coagulantes de sais de cálcio apresentam quantidades extras de cálcio, deste modo, pode-se estar utilizando GDL em misturas com sais de cálcio (LIU, 1997).

2.3 Parede Celular

A parede celular (Figura 4) é uma zona de fronteira entre cada célula e apresenta diversas funções tais como: rigidez, tamanho e formato das células, controle de crescimento de células durante o envelhecimento da parede, transporte de água através dos tecidos vegetais, proteção contra ataque de agentes patogênicos e controle no transporte intercelular, além de armazenar reservas alimentares (WALDRON & BRETT, 1996; BUCKERIDGE, TINÉ, SANTOS & LIMA, 2000; ZHONG & YE, 2007).

Figura 4 – Estrutura da parede celular vegetal



Fonte: Amabis & Martho (2006).

2.3.1 Composição da Parede Celular

Os polissacarídeos presentes na parede celular são estruturas extremamente complexas unidas por ligações intermoleculares (ligações iônicas, covalentes, pontes de hidrogênio e força de van der Waals). Embora os polissacarídeos que são encontrados nas plantas são formados por apenas 10 monossacarídeos, cada monossacarídeo pode existir em duas formas (piranose e furanose), e estes resíduos podem ser ligados por ligações glicosídicas em cada um dos três, quatro ou cinco grupos hidroxila disponíveis e por duas orientações (α ou β) (McDOUGALL *et al.*, 1996; BUCKERIDGE, TINÉ, SANTOS & LIMA, 2000).

Os polissacarídeos da parede celular são considerados um complexo de duas fases, a fase microfibrilar, composta principalmente por celulose, e a fase matriz, constituída por PNCs (polissacarídeos não-celulósicos). Os hemicelulósicos, que constituem a hemicelulose, se ligam através de pontes de hidrogênio com a celulose regulando a resistência e a porosidade da parede controlando a separação das microfibrilas (McDOUGALL *et al.*, 1996). A fase microfibrilar se diferencia da fase matriz devido a sua cristalinidade e composição química homogênea (WALDRON & BRETT, 1996; BUCKERIDGE, TINÉ, SANTOS & LIMA, 2000).

A fase microfibrilar é composta por estruturas extremamente finas e longas denominadas de microfibrilas, as quais são constituídas por moléculas de celulose, alinhadas paralelamente umas as outras. A fase matriz é uma camada não cristalina da parede celular; consiste numa grande variedade de polissacarídeos, proteínas e compostos fenólicos e sua composição pode variar em diferentes partes da parede celular, em diferentes tipos de células e em diferentes espécies. Esta variação não consiste apenas na proporção dos polímeros presentes, mas também na estrutura destes polímeros (WALDRON & BRETT, 1996; BUCKERIDGE, TINÉ, SANTOS & LIMA, 2000).

2.3.1.1 Celulose

É um homopolímero de moléculas de glicose unidas por ligações glicosídicas β-1,4 com grau de polimerização (resíduos de açúcares por molécula) de no mínimo 15000. As moléculas de celulose paralelas e adjacentes estão interligadas, por meio de pontes de hidrogênio, tanto intermoleculares quanto intramoleculares, formando microfibrilas muito resistentes. As fibras de celulose são formadas por regiões amorfas e cristalinas. A porção hidrolisável é denominada de amorfa e a porção resistente de cristalina (WALDRON & BRETT, 1996; DA-SILVA, FRANCO & GOMES, 1997; LEROUXEL, CAVALIER, LIEPMAN & KEEGSTRA, 2006).

2.3.1.2 Hemicelulose

Hemiceluloses são geralmente extraídas dos polissacarídeos da parede celular usando soluções alcalinas após a remoção da pectina (WALDRON & BRETT, 1996). Estes polissacarídeos apresentam ligações β-1,4 de xilose, manose ou glicose, podendo formar ligações de hidrogênio com a celulose. Xiloglucana é o principal componente presente na hemicelulose da parede celular primária dos vegetais (McDOUGALL *et al.*, 1996; LEROUXEL, CAVALIER, LIEPMAN & KEEGSTRA, 2006).

2.3.1.3 Polissacarídeos pectínicos

Este grupo é formado por polissacarídeos ricos em ácido galacturônico, ramnose, arabinose e galactose. Eles são característicos da lamela média e parede celular primária de dicotiledôneas e apresentam ligações covalentes entre si, além de se ligarem com celulose, fenóis e proteínas (WALDRON & BRETT, 1996; LEROUXEL, CAVALIER, LIEPMAN & KEEGSTRA, 2006).

2.3.1.4 Proteínas e glicoproteínas

A parede celular contém proteínas estruturais, onde a principal é a extensina, uma glicoproteína (50% de proteína) que apresenta um aminoácido incomum em sua composição, a hidroxiprolina (aproximadamente 40%) e grandes quantidades de lisina e serina, este último é capaz de fazer ligações cruzadas formando ligações covalentes intra e intermoleculares com demais constituintes da parede celular. A presença da hidroxiprolina torna fácil a identificação e localização destas proteínas (WALDRON & BRETT, 1996).

2.3.1.5 Lignina e outros compostos fenólicos

Lignina é um polímero fenólico formado pela polimerização de três alcoóis: álcool coniferil, álcool trans-p-cumaril e álcool trans-sinapil. Estas unidades de fenilpropano estão ligadas por um modelo irregular e tridimensional de éter e ligações carbono-carbono, onde um ou mais carbono pode ser aromático. O modelo irregular é decorrente da natureza não enzimática do processo de polimerização. Estes compostos tendem a fixar os polímeros, excluindo a água conferindo uma resistência ao vegetal. Além disso, a lignina pode apresentar ligações covalentes com polissacarídeos de forma direta com resíduos de açúcares ou indiretamente via ácido ferúlico esterificado (McDOUGALL *et al*., 1996; WALDRON & BRETT, 1996).

Outros compostos fenólicos podem ser encontrados, como por exemplo, o ácido ferúlico, o qual é esterificado com arabinose e galactose em pectinas, apresentando importante função nas ligações cruzadas nas moléculas de pectina. Em cereais onde a quantidade de pectina é baixa, o ácido ferúlico tem a

função de ligar a arabinose das arabinoxilanas. Estes ácidos normalmente são encontrados na forma de dímeros cíclicos.

Huisman, Schols e Voragem (1998) isolaram e caracterizaram polissacarídeos da parede celular da soja (*Glicine max*). A partir do resíduo desengordurado de soja, foram extraídos sólidos insolúveis em água. O isolamento destes sólidos resultou numa fração de 92% de polissacarídeos. Arabinose, galactose, ácidos urônicos e glicose (principalmente celulose) foram os principais constituintes do resíduo desengordurado da soja e também dos sólidos insolúveis extraídos, sendo então uma indicação de considerável presença de pectina.

Cadeias pequenas de açúcares como, frutose, sacarose, rafinose e estaquiose, são solúveis em água e foram recuperados na fração filtrada e retida durante o processo de obtenção dos sólidos insolúveis em água. Foi verificado que o resíduo desengordurado de soja continha 0,6% de frutose, 5,4% de sacarose, 0,8% de rafinose e 4,9% de estaquiose. Os teores de recuperação de sacarose (0%), rafinose (17%) e estaquiose (43%) detectados nas frações foram baixos. Foi observado também que o processo de isolamento resultou num extrato insolúvel em água de polissacarídeos muito similar ao resíduo desengordurado de soja, indicando que nenhum resíduo específico de açúcar foi removido durante o processo (HUISMAN *et al.,* 1998).

Seibel e Beléia (2008) analisaram fibras de cotilédones de soja e verificaram que a hemicelulose é o componente majoritário (59%) e a celulose o componente minoritário (8,5%), pois esta última fração está concentrada principalmente nas cascas de soja. Ouhida, Perez e Gasa (2002) observaram a presença de 10,7% de celulose nos cotilédones que continham as cascas. Em relação aos monossacarídeos, foram encontrados galactose, glicose e arabinose/ramnose.

Embora estudos quantifiquem os polissacarídeos presentes, poucos verificaram as influências do genótipo e condições ambientais na concentração dos polissacarídeos na parede celular. Deste modo, Stombaugh, Jung, Orf e Somers (2000) estudaram a influência destes fatores nos polissacarídeos e monossacarídeos da parede celular.

A pesquisa foi desenvolvida com 14 cultivares de soja. Os polissacarídeos foram quantificados após hidrólise das subunidades de

monossacarídeos através do método de determinação de fibra total de Uppsala. A concentração de polissacarídeos variou de 158 a 176 g/Kg entre as cultivares de soja. O genótipo da cultivar influenciou na concentração total dos polissacarídeos, porém as condições ambientais não influenciaram na concentração. No entanto, em relação à concentração de monossacarídeos, ambos os efeitos foram significativos. O efeito do genótipo foi significativo ($p \leq 0,05$) para as concentrações de fucose, mannose, arabinose, xilose, galactose e glicose. Efeitos genotípicos também foram observados no conteúdo de pectina (STOMBAUGH *et al*., 2000).

2.3.2 Hidrólise Enzimática da Parede Celular Vegetal

As propriedades físico-químicas de macromoléculas como proteínas e carboidratos, podem ser manipuladas por tratamentos químicos, mecânicos, enzimáticos ou térmicos. Os objetivos destas modificações são aumentar a funcionalidade e a disponibilidade de nutrientes, produzir hidrolisados com peptídeos definidos, isolar peptídeos fisiologicamente ativos e remover sabores ou odores assim como compostos tóxicos. A hidrólise enzimática é preferida em proteínas e carboidratos porque pode ser controlada, havendo uma alteração total, parcial ou específica, dependendo do interesse (SEIBEL & BELÉIA, 2009).

Em proteínas, os tratamentos térmicos e mecânicos causam desnaturação, resultando em menor solubilidade, perda de atividade biológica e aumento de interações proteína-proteína ou proteína com lipídeos, carboidratos e minerais. Estas interações também são ocasionadas por tratamentos químicos, que provocam modificações na polaridade, além de serem caros e não específicos, podem formar produtos tóxicos. A hidrólise enzimática é mais seletiva, melhora a disponibilidade de aminoácidos e os processos podem ser acelerados com maior quantidade de enzima, reduzindo tempos e custos operacionais. Devido ao maior controle na hidrólise, os produtos resultantes de modificações enzimáticas podem ser utilizados em dietas especiais (LIU, 1997; MARSMAN, GRUPPEN, MUL & VORAGEN, 1997; FISCHER, KOFOD, SCHOLS, PIERSMA, GRUPPEN & VORAGEN, 2001).

Seibel e Beléia (2009) hidrolisaram fibras de cotilédones de soja, utilizando Viscozyme L (complexo multienzimático de carboidrases de *Aspergillus*

aculeatus) e protease de *Bacillus licheniformis* e caracterizaram os hidrolisados solúveis e sólidos. Foi observado que as condições ideais de hidrólise para obter a maior solubilização dos carboidratos e proteínas foram: 200 µL de carboidrase/g de fibra alimentar, durante 12 horas sob agitação a 30 oC, e 150 µL de protease/g de fibra alimentar, durante 5 horas sob agitação a 55 oC. As frações sólidas da hidrólise com carboidrase tiveram, em média, 62% a mais de proteínas (45,5 g) às amostras antes da hidrólise (28,2 g), portanto, se há interesse em aumentar rendimento em proteínas por extração, a aplicação da carboidrase poderia extrair quantidades adicionais de proteínas. A fração solúvel apresentou 73% dos carboidratos (47,8 g) e 50% dos ácidos urônicos (8,5 g) da quantidade inicial das amostras (65 e 17,5 g, respectivamente).

O uso da protease solubilizou 54% do total de proteínas presentes nas amostras e ocorreu um aumento de 60% para 76% de fibras alimentares totais, sendo estas fibras, as frações majoritárias antes e depois da hidrólise, pois em relação às fibras solúveis os teores eram de 3,8 e 4,4, antes e depois da hidrólise, respectivamente (SEIBEL & BELÉIA, 2009).

Fischer *et al*. (2001) solubilizaram 76% dos carboidratos de farinha de soja após tratamento enzimático e verificaram que a extração incompleta é devido à complexa estrutura dos componentes da soja. Marsman *et al*. (1997) solubilizaram praticamente a metade dos ácidos urônicos presentes em farinha de soja, utilizando enzima para degradação de parede celular após 24 horas de hidrólise.

O uso de carboidrases na extração de proteínas tem sido aplicado desde 1997. Os polissacarídeos não amidolíticos estão localizados na parede celular de vegetais e podem limitar o acesso de enzimas endógenas a nutrientes, diminuindo sua disponibilidade. A parede celular da soja é constituída de 30% de pectina, 50% e hemicelulose e 20% de celulose. O uso de complexos multienzimáticos, como Viscozyme L, que contém carboidrases como arabinase, cellulase, gluconases, hemicellulase e xilanase (ANON, 2008), é capaz desintegrar tecidos da parede celular, através da ruptura das ligações entre polissacarídeos liberando celulose, hemicelulose e xilose e outros componentes intercelular como proteínas e compostos fenólicos.

Ansharullah e Colin (1997) estudaram métodos para aumentar a extração de proteínas em farelo de arroz. Foram estudadas as variáveis concentração de Viscozyme L (0-120 FBG) e Celluclast 1.5L (0-360 NC), tempo de incubação (1-5 h) e pH (3,8-5,4). Os experimentos foram conduzidos em diferentes temperaturas (40 e 50 oC). Os resultados indicaram que, em geral, a extração do nitrogênio foi maior quando o processo foi conduzido a 50 oC. Os autores observaram que a quantidade máxima de nitrogênio extraída, a 40 oC, ocorreu quando aplicada uma mistura de Viscozyme (120 FBG)/Celluclast (360 NC) em pH 3,8, com tempo de incubação de 5 horas, resultando na extração de 51,17% de proteínas. Quando utilizado 50 oC, e sob condições similares às anteriores, o uso de apenas Viscozyme (120 FBG) rendeu maior extração de N (57,89%), quando comparado ao uso da combinação das enzimas (54,42%) ou com apenas Celluclast (25,94%).

Comparando métodos alcalinos com enzimáticos na extração do N protéico, Ansharullah e Colin (1997) observaram que o uso de enzimas promoveu maior extração, em relação ao uso de álcalis. Quando aplicado pH 8,5; 9,0 e 9,5 foram obtidos 11,43; 32,28 e 46,98% de N, respectivamente. Porém com aplicação de enzimas em pH 7,0, foram obtidos 43,07 e 53,20% a 40 e 50 oC, respectivamente.

Tang *et al.* (2003) avaliaram a eficiência de três carboidrases na extração de proteínas de farelo de arroz desengordurado. Amilase, Viscozyme e Celluclast extraíram 45,4, 28,5 e 12,1% de proteínas nas concentrações de 11000 unidades de amilase, 160 FBG de Viscozyme e 700 EGU de Celluclast, respectivamente. Outras concentrações de Viscozyme também foram avaliadas: 0, 5, 10, 15, 20, 40 e 80 FBG, e foram obtidos os seguintes teores de extração protéica 14,1; 18,0; 20,2; 21,6; 24,5 e 28,3, respectivamente.

Guan e Yao (2008) estudaram os efeitos de Viscozyme L no aumento da extração de proteína em farelo de aveia. As condições ótimas foram definidas através de metodologia de superfície de resposta. Foram estudadas concentração (6–30 FBG), pH (3,0–5,0), tempo de incubação e (0,5–2,5 h) e temperatura (35–55 oC). As condições ótimas encontradas pelos autores para atuação de Viscozyme L foram 30 FBG/10 g farelo de aveia, pH 4,6, tempo de

incubação de 2,8 horas e temperatura de 44 oC; sob estas condições a extração foi de 56,2%, enquanto que pelo método alcalino (pH 9,5) a extração foi de 14,76%.

Deste modo, os autores comprovaram a eficiência no uso do método enzimático para aumentar a extração de proteínas.

Dueñas, Hernández e Estrella (2007) estudaram os efeitos das enzimas Tanase, α-Galactosidade, Fitase e Viscozyme na capacidade antioxidante de extratos metanólicos de farinha de lentilhas utilizando o teste DPPH. O uso das enzimas Viscozyme, α-Galactosidase e Tanase produziram um aumento na atividade antioxidante (EC50 = 2,16; 2,29 e 2,50, respectivamente) quando comparado a farinha controle (EC50 = 3,16). No entanto, o uso de Fitase provocou uma diminuição na atividade antioxidante (EC50 = 3,41) possivelmente devido à degradação do ácido fítico (AF) presente na amostra. Com a diminuição no teor de ácido fítico, aumenta a disponibilidade de cátions no meio que antes formavam quelatos com o AF, favorecendo a oxidação de diferentes componentes.

A aplicação de carboidrases em suspensão de soja pode ser uma boa alternativa para produção de tofus com propriedades antioxidantes e prebióticas. O uso de carboidrases tem se mostrado eficiente na extração de proteínas de soja, porém não há estudos onde enzimas tenham sido aplicadas para produção de tofus.

REFERÊNCIAS

AMABIS. J. M; MARTHO, G. R. **Fundamentos da Biologia Moderna.** São Paulo: Moderna, 4.ed, vol. único, 839p., 2006.

ANON. **Product sheet of Viscozyme L.** Novo Nordisk A/S, Enzymes process division, Bagsvaerd, Denmark, 2008.

ANSHARULLAH, J. A. H.; COLIN, F. C. Application of carbohydrases in extracting protein from rice bran. **Journal of the Science and Food Agriculture**, v.74, n.2, p. 141-146, 1997.

BAINY, E. M.; TOSH, S. M.; CORREDIG, M.; POYSA, V.; WOODROW, L. Varietal differences of carbohydrates in defatted soybean flour and soy protein isolate by-products. **Food Chemistry**, v.72, n.4, p. 664-672, 2008.

BARNES, S.; GRUBBS, C.; SETCHELL, K, D.; CARLSON, J. Soybeans inhibit mammary tumors in models of breast cancer. **Progress in Clinical and Biological Research**, v.347, p. 239-253, 1990.

BARNES, S.; KIRK, M.; COWARD, L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. **Journal of Agricultural and Food Chemistry**, v.42, n.11, p. 2466-2474, 1994.

BEDANI, R.; ROSSI, E. A. Isoflavonas: bioquímica, fisiologia e implicações para a saúde. **Boletim CEPPA**, v.23, n.2, p. 231-264, 2005.

BEHRENS, J. H.; DA SILVA, M. A. A. P. Atitude do consumidor em relação à soja e produtos derivados. **Ciência e Tecnologia de Alimentos**, v.24, n.3, p. 431-439, 2004.

BRASIL. **Resolução - CNNPA ANVISA, nº 14, de 28 de junho de 1978**, resolve padrão de identidade e qualidade para Farinha Desengordurada de Soja, Proteína Texturizada de Soja, Proteína Concentrada de Soja, Proteína Isolada de Soja e Extrato de Soja. Brasília, 2010b**.** Disponível em: <http://www.anvisa.gov.br>. Acesso em: 05 de Dezembro de 2010.

BUCKERIDGE, M. S.; TINÉ, M. A. S.; SANTOS, H. P.; LIMA, D. U. Polissacarídeos de reserva de parede celular em sementes. Estrutura, metabolismo, funções e aspectos ecológicos. **Revista Brasileira de Fisiologia Vegetal**, v.12, (Edição Especial), p.137-162, 2000.

CARRÃO-PANIZZI, M. C.; BERHOW, M.; MANDARINO, J. M. G.; OLIVEIRA, M. C. N. Environmental and genetic variation of isoflavone content of soybean seeds grown in Brazil. **Pesquisa Agropecuária Brasileira**, v.44, n.11, p. 1444-1451, 2009.

CARRÃO-PANIZZI, M. C.; BORDINGNON, J. R. Activity of beta-glycosidase and levels of isoflavone glucosides in soybeans cultivars affected by the environment. **Pesquisa Agropecuária Brasileira**, v.35, n.5, p. 873-878, 2000.

CARRÃO-PANIZZI, M. C.; SIMÃO, A. S.; KIKUCHI, A. Efeitos de genótipos, ambientes e de tratamentos hidrotérmicos na concentração de isoflavonas agliconas em grãos de soja. **Pesquisa Agropecuária Brasileira**, v.8, n.8, p. 897-902, 2003.

CIABOTTI, S.; BARCELLOS, M. F. P.; PINHEIRO, A. C. M.; CLEMENTE, P. R.; LIMA, M. A. C. Características sensoriais e físicas de extratos e tofus de soja comum processada termicamente e livre de lipoxigenase. **Ciência e Tecnologia de Alimentos,** v.27, n.3, p. 643-648, 2006.

CONAB. **Companhia Nacional de Abastecimento.** Décimo Segundo Levantamento de Grãos (Setembro de 2011). 2011. Disponível em: <http://www.conab.gov.br/conteudos.php?a =1253&t=2>. Acesso em: 11 out. 2011.

COWARD, L.; BARNES, N. C.; SETCHELL, K. D. R.; BARNES, S. Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. **Journal of Agricultural and Food Chemistry**, v.41, n.11, p. 1961-1967, 1993.

COWARD, L.; SMITH, M.; KIRK, M.; BARNES, S. Chemical modification of isoflavones in soyfoods during cooking and processing. **The American Journal of Clinical Nutrition**, v.68, n.6, p. 1486S- 1491S, 1998.

DA-SILVA, R.; FRANCO, C. M. L.; GOMES, E. Pectinases, hemicelulases e celulases, ação, produção e aplicação no processamento de alimentos: revisão. **Boletim SBCTA**, v.31, n.2, p. 249-260, 1997.

DUEÑAS, M.; HERNÁNDEZ, T.; ESTRELLA, I. Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity. **Food Chemistry**, v.101, n.1, p. 90-97, 2007.

EMBRAPA. **Empresa Brasileira de Pesquisa Agropecuária**. Soja em números (safra 2010/2011). 2011. Disponível em: <http://www.cnpso.embrapa.br/index.php?op_page =294 &cod_pai=17>. Acesso em: 11 out. de 2011.

ELDRIDGE, A. C.; KWOLEK, W. F. Soybean Isoflavones: Effect of Environment and Variety on Composition. **Journal of Agriculture and Food Chemistry**, v.31, n.2, p. 394-396, 1983.

FISCHER, M.; KOFOD, L. V.; SCHOLS, H. A.; PIERSMA, S. R. GRUPPEN, H.; VORAGEN, A. G. J. Enzymatic extractability of soybean meal proteins and carbohydrates: heat and humidity effects. **Journal of Agriculture and Food Chemistry**, v.49, n.9, p. 4463-4469, 2001.

FUKUTAKE, M.; TAKAHASHI, M.; ISHIDA, K.; KAWAMURAI, H.; SUGIMURA, T.; WAKABAYASHI, K. Quantification of genistein and genistin in soybeans and soybean products. **Food and Chemical Toxicology**, v.34, n.5, p. 457-461, 1996.

GÓES-FAVONI, S. P.; BELÉIA, A. D. P; CARRÃO-PANIZZI, M. C.; MANDARINO, J. M. G. Isoflavonas em produtos comerciais de soja. **Ciência e Tecnologia de Alimentos**, v.24, n.4, p. 582-586, 2004.

GUAN, X.; YAO, H. Optimization of Viscozyme L- assisted extraction of oat bran protein using response surface methodology. **Food Chemistry**, v. 106, n. 1, p. 345-351, 2008.

GUO, S.; ONO. T. The role of composition and content of protein particles in soymilk on tofu curding by glucono-δ-lactone or calcium sulfate. **Journal of Food Science,** v.70. n.4, p. C258-C262, 2005.

HUI, E., HENNING, S. M., PARK, N., HEBER, D., & GO V. L. W. Genistein and daidzein/glycitein content in tofu. Journal of Food Composition and Analysis, v. 14, n.2, p. 199-206, 2001.

HUISMAN, M. M. H.; SCHOLS, H. A.; VORAGEN, A. G. J. Cell wall polysaccharides from soybean (*Glycine max*.) meal. Isolation and characterization. **Carbohydrate Polymers**, v.37, n.1, p. 87-95, 1998.

HYMOWITZ, T.; WALKER, W. M.; COLLINS, F. I.; PANCZNER, J. Stability of sugar content in soybean strains. **Communications in Soil Science and Plant Analysis**, v.3, n.5, p. 367–373, 1972a.

HYMOWITZ, T.; WALKER, W. M.; COLLINS, F. I.; PANCZNER, J. Relationship between the content of oil, protein and sugars in soybeans seed. **Agronomy Journal**, v.64, n.2, p. 613-616, 1972b.

IZUMI, T.; PIKULA, M. K.; OSAWA, S.; OBATA, A.; TOBE, K.; SAITO, M.; KATAOKA, S.; KUBOTA, Y.; KIKUCHI, M. Soy Isoflavone aglycones are absorbed aaster and in higher amounts than their glucosides in humans. **The Journal of Nutrition**, v.130, n.7, p.1695-1699, 2000.

JACKSON, C. J. C.; DINI, J. P.; LAVANDIER, C.; RUPASINGHE, H. P. V.; FAULKNER, H.; POYSA, V.; BUZZELL D.; DEGRANDIS, S. Effects of processing on the content and composition of isoflavones during manufacturing of soy beverage and tofu. **Process Biochemistry**, v.37, n.10, p. 1117–1123, 2002.

KAO, T. H.; LU, Y. F.; HSIEH, H. C.; CHEN, B. H. Stability of isoflavone glucosides during processing of soymilk and tofu. **Food Research International**, v.37, n.9, p. 891–900, 2004.

KARIM, A. A.; SULEBELE, G. A.; AZHAR, M. E.; PING, C. Y. Effect of carrageenan on yield and properties of tofu. **Food Chemistry**, v.66, n.2, p. 159-165, 1999.

KIM, J. J.; KIM, H. S.; HAHN. J. S.; CHUNG, M. I. Changing soybean isoflavone composition and concentrations under two different storage conditions over three years. **Food Research International**, v.38, n.4, p. 435 – 444, 2005.

KOHYAMA, K.; SANO, Y.; DOI, E. Rheological characteristics and gelation mechanism of tofu (soybean curd). **Journal of Agricultural and Food Chemistry**, v.43, n.7, p. 1808-1812, 1995.

KUDOU, S.; FLEURY, Y.; WELTI, D.; MAGNOLATO, D.; UCHIDA, T.; KITAMURA, K.; OKUBO, K. Malonil isoflavone glycosides in soybeans seeds (*Gyicine max* [Merrill]). **Agriculture and Biological Chemistry**, v.55, n.9, p. 2227- 2233, 1991.

LAMARTINIERE, C. A.; ZHAO, Y. X.; FRITZ, W. A. Genistein: Mammary cancer chemoprevention, in vivo mechanisms of action, potential for toxicity, and bioavailability in rats. **Journal of Women's Cancer**, v.2, p. 11–19, 2000.

LEROUXEL, O.; CAVALIER, D. M.; LIEPMAN, A. H.; KEEGSTRA, K. Biosynthesis of plant cell wall polysaccharides — a complex process. **Current Opinion in Plant Biology**, v.9, n.6, p. 621–630, 2006.

LIU, K. **Soybeans: Chemistry, Technology and Utilization**. New York: Chapman & Hall, 537p., 1997.

MARSMAN, G. J. P.; GRUPPEN, H.; MUL, A. J.; VORAGEN, A. G. J. In Vitro Accessibility of Untreated, Toasted, and Extruded Soybean Meals for Proteases and Carbohydrases. **Journal of Agriculture and Food Chemistry**, v.45, n.10, p. 4088-4095, 1997.

McDOUGALL, G. J.; MORRISON, I. M.; STEWART, D.; HILLMAN, J. R. Plant cell walls as dietary fibre: range, structure, processing and function. **Journal of the Science of Food and Agriculture**, v.70, n.2, p. 133-150, 1996.

MIN, S.; YU, Y.; MARTIN, S. ST. Effect of soybean varieties and growing locations on the physical and chemical properties of soymilk and tofu. **Journal of Food Science: Food Chemistry and Toxicology**, v.70, n.1, p. C8-C12, 2005.

MORAES, R. M.; HAJ-ISA, N. M. A.; ALMEIDA, T. C. A.; MORETTI, R. H. Efeito da desodorização nas características sensoriais de extratos hidrossolúveis de soja obtidos por diferentes processos tecnológicos. **Ciência e Tecnologia de Alimentos**, v.26, n.1, p. 46-51, 2006.

MORAIS, A. A. C.; DA SILVA, A. L. **Soja: Suas Aplicações**. Rio de Janeiro: MEDSI, Médica e Científica. 259p., 1996.

MUSSATTO, S. I.; MANCILHA, I. M. Non-digestible oligosaccharides: A review. **Carbohydrate Polymers,** v.68, n.3, p. 587–597, 2007.

OUHIDA, I.; PÉREZ, J. F.; GASA, J. Soybean (*Glycine max.*) cell wall composition and availability to feed enzymes. **Journal of Agricultural and Food Chemistry**, v.50, n.7, p. 1933-1938, 2002.

PETERSON, T. G.; BARNES, S. Genistein and Biochanin A inhibit the growth of human prostate cancer cells, but not epidermal growth factor receptor tyrosine autophosphorylation. **Prostate**, v.22, n.4, p. 335-345, 1993.

QIANG, X.; YONGLIE, C.; QIANBING, W. Health benefit application of functional oligosaccharides. **Carbohydrate Polymers**, v.77, n.3, p. 435–441, 2009.

SEIBEL, N. F.; BELÉIA, A. D. P. Carboidratos das fibras de cotilédones e proteínas de produtos derivados de soja (*Glycine max* (L.) Merril). **Ciência e Tecnologia de Alimentos**, v.28, n.3, p. 607-613, 2008.

SEIBEL, N. F.; BELÉIA, A. D. P. Enzymatic hydrolyses of fibers from soy cotyledons and characterization of solid and soluble fractions. **Pesquisa Agropecuária Brasileira**, v.44, n.10, p. 1336-1345, 2009.

SILVA, M. S.; NAVES, M. M. V.; OLIVEIRA, R. B. De; LEITE, O. S. M. Composição química e valor protéico do resíduo de soja em relação ao grão de soja. **Ciência e Tecnologia de Alimentos**, v.26, n.3, p. 571-576, 2006.

STOMBAUGH, S. K.; JUNG, H. G.; ORF, J. H.; SOMERS, D. A. Genotypic and environmental variation in soybean seed cell wall polysaccharides. **Crop Science,** v.40, n.2, p. 408-412, 2000.

SUN, N.; BREENE, W. M. Calcium sulfate concentration influence on yield and quality of tofu from five soybean varieties. **Journal of Food Science**, v.56, n.6, p. 1604-1607, 1991.

TANG, S.; HETTIARACHCHY, N. S.; ESWARANANDAM, S.; CRANDALL, P. Protein extraction from heat-stabilized defatted rice bran: II. The role of Amylase, Celluclast, and Viscozyme. **Journal of Food Science**, v.68, n.2, p. 471-475, 2003.

TODA, K.; CHIBA, K.; ONO, T. Effect of components extracted from okara on the physicochemical properties of soymilk and tofu texture. **Journal of Food Science,** v.72, n.2, p. C108-C113, 2007.

TOMOMATSU, H. Health effects of oligosaccharides. **Food Technology**, v.48, n.10, p. 61-65, 1994.

VAN DER RIET, W. B.; WIGHT, A. W.; CILLIERS, J. J. L.; DATEL, J. M. Food chemical investigation of tofu and its by product okara. **Food Chemistry**, v.34, n.3, p. 193-202, 1989.

WALDRON, K. W.; BRETT, C. T. **Physiology and Biochemistry of plant cell walls**. New York: Chapman & Hall, 245 p, 1996.

WANG, H. J; MURPHY, P. A. Isoflavone content in commercial soybean foods. **Journal of Agriculture and Food Chemistry**, v.42, n.8, p. 1666-1673, 1994a.

WANG, H. J; MURPHY, P. A. Isoflavone composition of american and japanese soybeans in Iowa: effects of variety, crop year, and location. **Journal of Agriculture and Food Chemistry**, v.42, n.8, p. 1674-1677, 1994b.

WANG, H. J.; MURPHY, P. A. Mass balance study of isoflavones during soybean processing. **Journal of Agricultural and Food Chemistry**, v.44, n.8, p. 2377-2383, 1996.

ZHONG, R.; YE, Z. Regulation of cell wall biosynthesis. **Current Opinion in Plant Biology**, v.10, n.6, p. 564–572, 2007.

3 RESULTADOS E DISCUSSÃO

Os resultados obtidos estão redigidos na forma de três artigos científicos, conforme segue.

3.1 ARTIGO CIENTÍFICO A

O artigo a seguir foi submetido ao periódico ***Food Science and Technology International***.

VISCOZYME L ACTION ON SOY SLURRY AFFECTS CARBOHYDRATES AND ANTIOXIDANT PROPERTIES OF SILKEN TOFU

VISCOZYME L ACTION ON SOY SLURRY AFFECTS CARBOHYDRATES AND ANTIOXIDANT PROPERTIES OF SILKEN TOFU

Summary
This study investigated the enzymatic treatment of soy slurry using Viscozyme L to hydrolyze carbohydrates. The optimum temperature of Viscozyme L action was at 55 oC. The increase of glucose and galactose content in tofu (1.36 and 0.19 g per 100 g, respectively) confirmed Viscozyme activity on soy slurry when compared to the control. The treated tofu had more total phenolics than the control (173 and 161 mg gallic acid equivalents per 100 g freeze-dried tofu, respectively) and higher antioxidant activity by the ABTS and DPPH test. Total reducing sugar (glucose equivalents) content in treated tofu was approximately 4 times higher than in the control under the optimum conditions (30 Fungal Beta-Glucanase units per 10 g solids, 55 oC, 30 min). The tofus differed in the sensory analysis for soy odour and surface uniformity, but there was no preference for one over the other.

Keywords: Reducing sugars. Soy products. Sensory analysis.

1 INTRODUCTION

Presently the use of foods that promote a state of wellbeing, better health and a claim of reduction of the risk of chronic diseases have become popular as the consumers are becoming more health conscious. In Brazil the functional food market is still incipient, however with a great potential, considering availability of natural resources, the productive capacity of the local industry and the size of the consumer market.

Soybean is a commodity with many derived industrialized food products with associated health claims that have been widely disseminated by the media in recent years. The inclusion of soy and its derivatives (soymilk and tofu) as part of daily diet is recommended because they contribute to provide the nutrients necessary for development, growth and maintenance of the body, and provide components, such as natural antioxidants that assist in the physical well-being, improve the functioning of the body and probably preventing chronic degenerative diseases (Liu, 1997).

Glucono-δ-lactone (GDL), an acid precursor is used in making silken‖ and softer tofus. Pasteurized pre-cooked soymilk is mixed with GDL and the resulting mixture is filled into the retail containers. The containers are heated in a water bath at

80 – 90 oC for 40 – 60 min, and the gradual release of gluconic acid from GDL hydrolysis induces protein gelation with minimal syneresis. Without removing the whey from the curd, GDL-coagulated silken tofu has a custard-like texture and smooth mouthful (Liu, 1997).

Sugars, especially sucrose contribute to the sweetness of soymilk and tofu. Total sugar content has been reported to range from 4.1% to 15.1% of the dry weight in soybeans. Sucrose is the primary component comprising up to 60% of the total sugars. The other two sugar components of soybeans seeds are stachyose and raffinose, which are not digestible and are considered to be a cause of flatulence associated to consumption soybean foods (Liu, 1997).

The biotechnological procedure of enzyme addition to legume flours have been recognized in the food industry and the main objective is to improve the utilization of nutrients from raw materials. Treatment for protein hydrolysis with proteases is common, but less studied is the hydrolysis of carbohydrates in soybeans by carbohydrases. The addition of the carbohydrase in soy slurry before obtaining the soymilk may result in tofu with technological properties and composition different from the traditional tofu. Due to the presence of this enzyme, insoluble polysaccharides could be partially hydrolyzed producing sugars or oligosaccharides, which would be transferred to the tofu, possibly changing the composition and texture. The hydrolysis of soybean insoluble polysaccharides can also facilitate protein and antioxidant release and sugars like glucose and galactose that would increase the sweetness of soy soymilk and tofu.

The non-starch polysaccharides are located in the cell wall and can limit the access of human‘s endogenous enzymes to nutrients reducing their bioavailability. The use of multienzymatic complexes, such as Viscozyme L, which contains cellulases, arabinases, hemicellulases, gluconases and xylanases, causes the rupture of the cell walls, favouring the extraction of useful compounds (protein and sugars) from the vegetable tissues (Dueñas, Hernández, and Estrella, 2007). However, investigation of Viscozyme L action, an enzyme approved for food use, on soy slurry and tofu has not been previously reported.

The objectives of this study were to apply an enzymatic treatment to soy slurry using Viscozyme L to hydrolyze insoluble carbohydrate and verify the effects of this enzyme on physicochemical and sensorial characteristics of silken tofu.

2 MATERIAL AND METHODS

Chemical and material

The chemical reagents 1,1-diphenyl-2-picryhydrazyl radical (DPPH˙+), 2,2‘-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS+), 2,4,6-tripyridyl-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich. Viscozyme L (from *Aspergillus aculeatus*) was a gift from Novozymes Latin American Inc. (Araucária, PR- Brazil). The activity of Viscozyme L was 100 Fungal Beta-Glucanase Units (FBG) g-1, in which 1 FBG is the amount of enzyme required under the standard conditions (30 oC, pH 5.0 and 30 min reaction time) that hydrolyzes barley β-glucan to reducing carbohydrates, corresponding to 1 μmol glucose min-1.

The soybean variety BRS 267 (large grains), crop 2007/2008 (EMBRAPA – Paraná, Brazil), was used to prepare the tofus. The antifoam agent was supplied by Prosabor (São Paulo-Brazil) and GDL (Sigma) was used as coagulant agent. All the other reagents were of analytical grade. The use of cultivar BRS 267 can improve the sensory quality of soy products like tofu and soymilk due the high sucrose content (5.96 g per 100 g), and could masking astringency, beany flavour and other flavourful compounds such as saponins and isoflavones (Oliveira et al., 2010).

Production of the soy slurry

Soybeans (approximately 150 g) were soaked in 1500 ml distilled water at room temperature for 16 h (soybean: water = 1:10). The soaked soybeans were drained, rinsed and ground with 1000 mL distilled hot water (98 oC) in a blender for 3 min at high speed. Antifoam agent (1.0 mL) was added to the slurry during grinding.

Enzymatic treatment of soy slurry

To a 200 mL portion of slurry 0.5 mL of Viscozyme L (30 FBG per 10 g solids) was added. The slurries containing the enzyme were incubated in a water bath at different temperatures (25, 35, 45, 55 and 65 oC) for 30 min. Then the enzyme was deactivated by heating (98 oC) for 10 minutes.

According to Novozymes Inc., the temperature range of the enzyme activity is from 25 to 55 oC on β- glucanase (endo-1,3(4)-) (Anon, 2008). To test the best temperature (25, 35, 45, 55 or 65 oC) for Viscozyme L action, a 30 minutes fixed hydrolysis time was used with an enzyme concentration of 30 FBG per 10 g solids (Guan and Yao, 2008).

Preparation of soymilk and tofu at optimum conditions

Tofu was prepared by a modification of the methods proposed by Min, Yu, and Martin (2005) and Shen et al. (1991). Viscozyme L (2.5 mL, 30 FBG per 10 g solids) was added to 1000 mL portion of soy slurry. After incubation at 55 oC for 30 min, the soy suspension was boiled (98 oC, 10 min) and filtered manually with a muslin cloth to obtain soymilk.

To a 400 mL portion of soymilk (45 oC) 10 mL of GDL (0.02 M) was added and placed into a plastic container which was transferred to a water bath (Marconi, MA 127) and left at 85 oC for 60 min. The yield of tofu was calculated as the weight of fresh tofu obtained from a specified amount of the soymilk (400 mL) used for its preparation. Tofus without the enzyme treatment (control) were produced in the same way, excluding only the step of enzyme addition. The tofus were frozen and lyophilized (0.040 mbar and -50 oC) in a freeze-dryer (Alpha 2-4 LD Plus, Christ) to determine sugars, antioxidant activity and total phenolics. The moisture content of lyophilized tofu with enzyme and control was 3.6% and 2.8%, respectively.

Quantification of reducing sugars

Extraction of reducing sugars followed the method of Leonel and Cereda (2002) and was determined by the method of Somogyi (1945) by

spectrophotometry with readings at 520 nm (UV-VIS Spectrophotometer, Cintra 20) and the results were expressed in glucose equivalents (GluE) using a standard curve of glucose solution (100 µg mL-1) with a range of 10 to 100 µg.

Sugars and uronic acids quantification

The concentrations of galactose and raffinose were determined following the raffinose-series oligosaccharides assay procedure (Megazyme K-RAFGA 10/04). Glucose was determined spectrophotometry using the Kit Glucose Bio Liquid (LABORCLIN). Uronic acids were determined by m-hydroxydiphenyl method according to Kintner and Van Buren (1982). Sodium tetraborate (0.0125 M) was prepared in concentrated sulphuric acid and the 0.15% solution of m-hydroxyphenyl was prepared in 0.5% sodium hydroxide. The results of sugar and uronic acids were expressed in g per 100 g of freeze-dried material.

Antioxidant activity and total phenolics

The extracts were obtained according to the method used by Shih, Yang, and Kuo (2002) with some modifications. One-gram sample of lyophilized tofu was extracted with 20 mL of 80% ethanol in water in a shaking incubator at 25 oC, 100 rpm for 24 h. The supernatant of the centrifuged sample (3000 x *g*) was separated and to the precipitate 10 mL of 80% ethanol was added and centrifuged again. The filtrates (30 mL) were concentrated to 10 mL at 40 oC and were stored at -22 oC for antioxidant activity determination.

For ABTS assay, the procedure followed the method of Thaipong et al. (2006). The standard curve was linear between 100 and 2000 µM Trolox. The DPPH assay was according to the method of Brand-Williams, Cuvelier, and Berset (1995). The standard curve was linear between 100 and 1000 µM Trolox. The FRAP assay was according to Benzie and Strain (1996). All results were expressed in µmol Trolox equivalents (TE) per g freeze-dried tofu.

Total phenolic content was determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959). The absorbance was measured at 760 nm using a spectrophotometer (UV-VIS spectrophotometer, Cintra

20) and the results were expressed in mg gallic acid equivalents (GAE) per 100 g freeze-dried tofu) using a gallic acid (0.1 – 0.6 mM) standard curve.

Physicochemical analyses

Moisture content of slurries and tofus was determined by drying a weighed amount of sample to a constant weight in a vacuum oven (60 oC). The ash content was determined as described in AOAC (1998) using incineration followed by calcination of the sample at 550 ºC. Protein was determined by micro Kjeldahl method (AOAC, 1998). Calcium and magnesium were determined by atomic absorption methods (Atomic absorption spectrometry GBC 932AA), after digestion in nitropercloric solution (HNO3:HClO4 /4:1) and appropriate dilution.

Texture and tofu colour analyses

Surface colour of tofus, expressed in L* (lightness) and b* (component yellow-blue), according to the CIE Lab definition were measured using BYK colorimeter (Gardner Lab Inc., USA) equipped with the light source D65 and observation angle of 45o/0o with opening of 11 mm.

Textural properties of tofu were evaluated with TA.XT2i Texture Analyzer (Stable Micro Systems, Goldaming, Surrey, UK). Cylindrical samples of tofu (30 mm diameter, 22 mm height) were compressed to 73% deformation. The parameter setting and operation of the instrument were accomplished through a PC with Texture Expert software version 1.19. A cylindrical aluminium probe (P 35) was used with the pre-test, test and post-test speeds were set to 2, 2 and 4 mm s-1, respectively (Karim et al., 1999).

Sensory evaluation

The tofus were served in coded closed bottles of 40 mL at approximately 10 oC, and opened at the time of sensory evaluation; the tests occurred with white lighting.

The triangle test was conducted with 47 panellists who were graduate students, staff and faculty members from the Departamento de Ciência e Tecnologia de Alimentos of Universidade Estadual de Londrina (DCTA/UEL). The samples were offered simultaneously in different possible combinations and present at random to the panellists to examine (appearance, aroma, taste and texture) and identify the odd sample (Stone and Sidel, 2004).

A descriptive analysis was carried out in the sensory laboratory at EMBRAPA SOJA (Paraná-Brazil). Nine different descriptive terms were used: cream colour, surface uniformity, soy odour, soy flavour, acid taste, bitter taste, astringency, firmness and succulence. The scale was a 9 cm line with words anchors located 0.5 cm from each end. The scale direction goes from left to right with increasing intensity. Ten trained panellists in descriptive analysis of tofu participated in the test. The samples were offered sequentially in random order. Replicates (3 times) of tofu samples were evaluated in different sessions separated by a 1day interval (Stone and Sidel, 2004).

A paired preference test was applied with 78 potential consumers who were graduate students, staff and faculty members from DCTA/UEL. The samples were offered in random order (Stone and Sidel, 2004).

Statistical analysis

All experiments and analysis were performed in triplicate. The texture and colour analysis were carried in six replicate. Analysis of variance (ANOVA) was conducted for every data collected, using Statistica 7.0 for Windows (StatSoft Inc.,USA). Differences among the mean values were also determined using the Tukey test and Student *t*-test. A significant level was defined as a probability of 0.05. The sensory results from triangle test was analyzed using binomial distribution tables (one-tailed, $p = 1/3$). The sensory results from paired preference test were analyzed using binomial distribution table (two-tailed, $p = 1/2$). The descriptive analysis results were analyzed by ANOVA, *F* test and Student *t*-test using Statistical Analysis System Programme 8.2 (SAS Institute Inc, USA).

3 RESULTS AND DISCUSSION

Characterization and determination of conditions for Viscozyme L action

Protein and minerals like calcium and magnesium (coagulants) can influence the final quality of the product, thus it was necessary to know the amount of these compounds presents in the enzyme because they could interfere in the process of coagulation. The calcium, magnesium and protein were found to be 69.6 mg mL-1, 134.2 mg mL-1 and 7.9 g per 100 mL, respectively. The pH of the enzyme was 5.01. These results indicated that the quantity of minerals was low due to the little amount of enzyme used in the processing (2.5 mL per 1000 mL soy slurry) and would not influence in the coagulation process because the amount of calcium and magnesium necessary to coagulate 1000 mL of soymilk is approximately 800 and 500 mg respectively, when used in the form of sulphate salts (CaSO4.2H2O, MgSO4.7H2O) (Liu, 1997; Prabhakaran, Perera, and Valiyaveettil, 2006).

The pH optimum for Viscozyme L action is from 3.5 to 5.5 (Anon, 2008). The pH of soy slurry is 6.69 (25 oC), and we did not study the influence of pH on enzyme action because it was previously established to work with the original pH of the slurry.

The use of the enzyme in soy slurry at 55 oC increased the content of reducing sugar (GluE) up to six times when compared to the control (Table 1). There was an increase in reducing sugar content as the temperature increased in the optimum temperature range (25 to 55 oC), while at 65 °C a decrease was observed due to some enzyme inactivation. There was no difference between treatment at 35 and 65 oC ($p > 0.05$) (Table 1). Reducing sugar production was highest at 55 oC (600 mg GluE per 200 mL soy slurry) and this is the maximum temperature determined by Novozymes Inc. for action on barley β-glucan (Anon, 2008).

Table 1 – Levels of reducing sugars (as glucose equivalents in mg per 200 mL) in the treated soy slurry and control.

Temperature (°C)	Treated soy slurry (30 FBG per 10 g solids)	Control soy slurry
25	458.29 ± 28.88^{d}	102.45 ± 7.85^{a}
35	494.64 ± 30.54^{c}	102.70 ± 7.63^{a}
45	562.31 ± 28.52^{b}	103.88 ± 4.80^{a}
55	600.65 ± 30.45^{a}	100.86 ± 7.36^{a}
65	501.08 ± 39.20^{c}	104.68 ± 4.89^{a}

Means values in the same column followed by different letters are different (Tukey, $p \leq 0.05$).

Effects of Viscozyme L in tofu composition

After obtaining the best conditions for Viscozyme L action according to the preliminary studies, application of Viscozyme L for tofu preparation using the kanetsu-shibori method was tested. Methods of making soymilk are roughly grouped into 2 kinds, ―nama-shibori‖ and ―kanetsu-shibori‖. In the nama-shibori method, raw soymilk is processed by squeezing the slurry and then heating. In contrast, soymilk is obtained by squeezing the slurry after heating in the kanetsu-shibori method, which is used by most tofu manufacturers in Japan. This method was chosen because the enzyme could act on full homogenized soy slurry, resulting from grinding the grains and not only in the separated soymilk without presence of the insoluble material (okara), rich in polysaccharides. Besides, silken tofu product was chosen because the products of the hydrolysis are probably water soluble. In a soft tofu (pressed) these sugars would be eliminated in the whey. Reducing sugar content in the tofu after enzyme hydrolysis was approximately 4 times higher than in the control tofu (Table 2).

The proximate chemical composition of tofu with and without enzyme treatment differed for protein content that was higher for the control tofu while lipids were lower (Table 2). The tofu composition was generally consistent with the literature report (Shen et al., 1991; Liu, 1997). The tofu yield was 392 g per 400 mL soymilk, much higher than tofus produced by Prabhakaran, Perera, and Valiyaveettil

(2006) (193 to 246 g per 500 mL soymilk), because the whey was not separated during the production of silken tofu.

Table 2 – Proximate composition and yield of treated and control tofu.

Analysis	Treated tofu	Control tofu
Moisture (%)	90.79 ± 0.03^{a}	90.36 ± 0.03^{b}
Ash (%)	0.56 ± 0.01^{a}	0.55 ± 0.00^{a}
Proteins (%)	4.67 ± 0.04^{a}	5.35 ± 0.48^{b}
Lipids (%)	0.86 ± 0.01^{a}	0.70 ± 0.00^{b}
Carbohydrates (%)	3.10	3.03
Reducing sugars (mg g^{-1})	3.13 ± 0.09^{a}	0.79 ± 0.00^{b}
Yield (g)	392	392

Means values in the same row followed by the same letters are not different (Student *t*-test, $p > 0.05$). Totals carbohydrates were obtained by difference. Yield was calculated from 400 mL of soymilk.

The firmness of treated tofu was lower than the control (Table 3). This softer texture may be related to compounds released by hydrolysis of insoluble carbohydrates from the soy slurry. Without removing the whey from the curd, GDL-coagulated silken tofu has a custard-like texture and smooth mouthful. These results are consistent with the results proposed by Gu, Campbell, and Euston (2009) where the soy protein sugar gels, especially the reducing sugars gel, were softer than control gel (without sugar). They also observed that the reducing sugars gels were more soluble than the control, with increased water holding capacity. This trend could be due to the protective effects of sugars against protein denaturation when heat treatment occurred at pH 6.9, which resulted in decreased protein precipitation upon GDL acidification resulting in softer gels.

Good quality tofu is white or light yellow in colour, which will depend of hilum and cotyledon colour. Traditionally, soymilk and tofu manufactures prefer large-seeded soybeans with light seed coat and clear hilum (Liu, 1997). Reducing sugars can modify the colour of the tofu with the heating necessary for coagulation,

so the determination of this parameter was necessary to verify the influence of Viscozyme L in tofu colour (Table 3).

Table 3 – Texture and tofu colour parameters.

Types of tofu	Firmness	CIE Lab values	
		L*	b*
Treated tofu	3.98 ± 0.28[b]	85.93 ± 0.36[a]	14.16 ± 0.19[a]
Control tofu	4.81 ± 0.32[a]	85.68 ± 0.52[a]	14.14 ± 0.46[a]

Means values in the same column followed by the same letters are not different (Student *t*-test, $p > 0.05$). Each value represented a mean value of six determinations. L* (lightness) and b* (component yellow-blue).

All of the tofu samples prepared in this experiment were light yellow in colour. Noh et al. (2005) characterized by the Hunter system the tofu colour: the b and L values were from 11.8 to 12.5 and 82.7 to 83.3, respectively, featuring a high brightness. Regarding the colour parameters of tofu analyzed and compared with quality parameters established by Noh et al. (2005), the tofus produced with enzyme treatment were in agreement with ideal colour for tofus. CIE Lab colorimetric readings also indicate no differences ($p > 0.05$) in L* and b* values as shown in Table 3. No development of yellow or brown colour was noticeable in any of ours samples. This means that Viscozyme L action had no effect on the colour of tofu. Gu, Campbell, and Euston (2009) reported no visually noticeable development of yellow or brown colour in any of the gel samples with reducing sugars like glucose and lactose.

The concentrations of glucose, galactose and the sum of raffinose and galactose are in Table 4. The increase of glucose and galactose content in treated tofu (1.36 and 0.19 g per 100 g, respectively) demonstrated activity in the degradation of polysaccharides releasing simple sugars. The glucose content corresponds to 43% of the total content of reducing sugars in treated tofu. It was observed that the amount of raffinose present in the treated tofu was lower than in the control tofu; so the compound was probably hydrolyzed in the enzymatic treatment.

Table 4 – Sugar composition (g per 100g) and uronic acids (µg g-1) of freeze-dried tofu.

Sugar	Treated tofu	Control tofu
Raffinose	4.04 ± 0.01^{b}	4.58 ± 0.13^{a}
Galactose	0.19 ± 0.00^{a}	0.09 ± 0.01^{b}
Glucose	1.36 ± 0.02^{a}	0.12 ± 0.00^{b}
Uronic acids	3.88 ± 0.26^{a}	3.13 ± 0.26^{b}

Means values in the same column followed by different letters are different (Student *t*-test, $p \leq 0.05$).

Mature soybeans contain trace amounts of monosaccharides, such as glucose and arabinose, and measurable amount of di and oligosaccharides, with sucrose in the range of 2.5–8.2%, raffinose 0.1–0.9%, and stachyose 1.4–4.1% (Bainy et al., 2008; Jiménez-Escrig, Serra, and Rupérez, 2010). Little is known about the cellular location of oligosaccharides but it is known that they are not in the cell wall (Liu, 1997). Then the action of Viscozyme L on insoluble carbohydrates did not originated any raffinose, which explains the higher content in control tofu, but it could have been responsible for the lower raffinose content in the treated tofu.

Raffinose and stachyose are the galactosyl derivatives of sucrose, the former with one and the latter with two moieties of galactose attached to sucrose via α 1→6 glycosidic linkage, they are referred as raffinose family oligosaccharides (RFOs). RFOs, unlike sucrose, are not digested in the human gastrointestinal tract and as result they pass to the lower gut, where they are digested by the intestinal microflora. In the process, flatulence-inducing gases, namely, CO2, CH4, and H2S, are liberated, causing abdominal discomfort and diarrhoea. The consumption of tofu treated with Viscozyme L could decrease this undesirable effect because there was a reduction of 12% of the raffinose content compared to the control.

The level of uronic acid in tofu with enzyme treatment was 3.9 µg g-1 whereas control tofu had 3.1 µg g-1 (Table 4), confirming the Viscozyme L action on pectin, although Viscozyme L being a multi-component carbohydrase does not have a pectinase activity.

Several studies report the use of carbohydrases (Viscozyme L and Celluclast 1.5 L) to increase the extraction of proteins from soybean cotyledon fibres

(Seibel and Beléia, 2009), oat meal (Guan and Yao, 2008), rice bran (Ansharullah and Colin, 1997; Tang et al., 2003), but these results were not observed in our study. Seibel and Beléia (2009) increased protein extraction (62%) from soybeans cotyledon fibres after Viscozyme action but after an alkaline extraction. It was expected that with the application of Viscozyme L in soy slurry, the protein would be released due fibre hydrolysis and transferred to tofu, but it was not observed; probably the short action time (30 min) of carbohydrase and the different pH (6.9) to the ideal (3.5-5.5).

New experiments were undertaken with the aim of increasing the level of protein in tofus. The enzyme incubation time was changed from 30 to 180 minutes, keeping the concentration of enzyme (30 FBG per 10 g solids) and temperature (55 oC). The amount of protein (5.09%) was similar to the tofus characterized in Table 2, but the reducing sugar increased to 6.17 mg GluE g-1 when compared to enzyme treated tofu 3.13 mg GluE g-1 with 30 min of incubation and control 0.79 mg GluE g-1. This result confirms the importance of alkaline conditions for the extraction of proteins and probably, the influence of pH in enzyme action.

Antioxidant activity and total phenolics

Tofu extracts with and without Viscozyme L differed ($p \leq 0.05$) in their total phenolic content (TPC) (Table 5). The TPC of tofu with and without enzymatic treatment was 173 and 161 mg GAE per 100 g freeze-dried tofu, respectively.

Table 5 – Total phenolics and antioxidant activity of tofu.

Analysis	Treated tofu	Control tofu
ABTS	6.41 ± 0.39^a	5.86 ± 0.27^b
DPPH	2.59 ± 0.28^a	2.13 ± 0.13^b
FRAP	5.11 ± 0.28^a	5.06 ± 0.35^a
Total phenolics	172.60 ± 6.38^a	161.50 ± 8.68^b

Means values in the same row followed by different letters are different (Student *t*-test, $p \leq 0.05$). Total phenolics content expressed in mg gallic acid equivalents per 100 g freeze-dried tofu. Results expressed in µmol Trolox equivalents per g freeze-dried tofu.

The tofu extracts differed in DPPH and ABTS ($p \leq 0.05$) values (Table 5). Tofu treated with the enzyme had 6.4 and 2.5 μmol TE per g freeze-dried tofu for ABTS and DPPH, respectively; whereas the control tofu these values were lower (5.8 and 2.1 μmol TE per g freeze-dried tofu, respectively). These results suggest that Viscozyme L acted on the cell wall and could release phenolic compounds increasing the antioxidant activity of the product. Dueñas, Hernández, and Estrella (2007) studied the effect of the enzymes tannase, α-galactosidase, phytase and viscozyme on the phenolic composition of lentil flours. The treatments with viscozyme (EC50 = 2.16), α-galactosidase (EC50 = 2.29) or tannase (EC50 = 2.50) produce an increase in the antioxidant activity when compared to raw lentils (EC50 = 3.16). A smaller EC50 value corresponds to a higher antioxidant activity. In contrast to other tests of total antioxidant activity, the FRAP values of tofu extracts were not differed ($p > 0.05$). While FRAP mechanism is totally by electron transfer, DPPH and ABTS mechanisms can be by electron or H atom transfer; the radicals may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer. Then, the mechanism will depend of type bioactive compounds present in extract and their availability to donate H atom to stabilize the free radical.

Sensory characteristics

The panellists in the triangle test detected that the samples were different ($p \leq 0.05$); thus a descriptive analysis was performed to determine which attributes in the samples differed.

The samples were characterized mainly by middle cream colour (5.3) and astringency (4.3), uniform surface (7.5), low firmness (1.7) and bitter taste (2.1) (Table 6). The tofus were different ($p \leq 0.05$) just in two attributes. The treated tofu surface was less uniform (7.3) and had more intense soy odour (4.2) than control (7.7 and 3.2, respectively). The surface uniformity is related to the presence of air bubbles caused by formation of foam during tofu processing although antifoam was used.

Table 6 – Effect of enzymatic treatment on sensory characteristics of tofu.

Tofu	Cream Colour	Surface uniformity	Soy odour	Soy flavour	Acid taste	Bitter taste	Astringency	Firmness	Succulence
Treated	5.3[a]	7.3[b]	4.2[a]	3.0[a]	5.2[a]	1.9[a]	4.3[a]	1.6[a]	5.5[a]
Control	5.2[a]	7.7[a]	3.2[b]	2.7[a]	4.7[a]	2.2[a]	4.3[a]	1.8[a]	5.7[a]

It was expected that the greater amount of glucose equivalents in treated tofu would result in a less intense flavour of soy, but this was not observed and may be related to increased intensity of acid taste in the sample. This attribute is result of the presence of GDL which was used as coagulant in the soymilk. Mahfuz et al. (2004) verified that the astringent sensation of soymilk disappeared after tofu curd formation as result of the addition of coagulant. The researchers used GDL, $CaCl_2/CaSO_4$ and $MgCl_2$ as coagulants and verified that the astringent sensation was higher on GDL tofu curd. Phytic acid plays an important role in tofu curd formation, interacting with Mg or Ca coagulant. However the GDL coagulant has less effect with phytic acid during coagulation and there is higher concentration of free phytate than when using Ca or Mg coagulant. Panel members expressed strong astringency in a 1% K-phytate solution whereas there no bad taste in Ca phytate solution of the same concentration, which would help explain the astringent sensation on GDL tofu curd.

Although the tofus were different by triangle test and differed in some attributes like appearance and odour, there was no preference ($p > 0.05$) for one over the other tofu.

CONCLUSIONS

Reducing sugar content in enzyme treated tofu was 4 times higher than control tofu. The increase of glucose and galactose content in treated tofu demonstrated enzymatic activity in the hydrolysis of polysaccharides releasing simple sugars. The use of Viscozyme L on the soy slurry resulted in increasing antioxidant activity in the produced tofu due to released phenolics compounds, as well as higher antioxidant activities as determined by ABTS and DPPH tests. Although the tofus differ from each other, there was no preference ($p > 0.05$) for one over the other by sensory analysis. The consumption of tofu treated with Viscozyme L could decrease the undesirable effect of flatulence because there was a reduction of the raffinose content compared to the control.

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REFERENCES

Anon. (2008). **Product sheet of Viscozyme** *L*. Novo Nordisk A/S, Enzymes process division. Bagsvaerd, Denmark.

Ansharullah, J.A.H. and Colin, F.C. (1997). Application of carbohydrases in extracting protein from rice bran. **Journal of the Science and Food Agriculture,** v.74, n.2, p. 141-146.

AOAC (1998). **Official Methods of Analysis**, 20th edn. Arlington, Virginia, USA: Association of Official Analytical Chemists.

Bainy, E.M., Tosh, S.M., Corredig, M., Poysa, V. and Woodrow, L. (2008). Varietal differences of carbohydrates in defatted soybean flour and soy protein isolate by-products. **Carbohydrate Polymers**, v.72, n.4, p. 664–672.

Benzie, I.F.F. and Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant powerǁ: The FRAP assay. **Analytical Biochemistry**, v.239, v.1, p. 70–76.

Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Food* **Science and Technology**, v.28, n.1, p. 25-30.

Dueñas, M., Hernández, T. and Estrella, I. (2007). Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity. **Food Chemistry**, v.101, n.1, p. 90-97.

Gu, X., Campbell, L.J. and Euston, S.R. (2009). Influence of sugars on the characteristics of glucono-δ-lactone-induced soy protein isolate gels. **Food Hydrocolloids***,* v.23, n.2, p. 314–326.

Guan, X. and Yao, H. (2008). Optimization of Viscozyme L- assisted extraction of oat bran protein using response surface methodology. **Food Chemistry**, v.106, n.1, p. 345-351.

Jiménez-Escrig, A., Serra, M.T. and Rupérez, P. (2010). Non-digestible carbohydrates in Brazilian soybean seeds [*Glycine max* (L.) Merril]. **International Journal of Food Science and Technology**, v.45, n.12, p. 2524–2530.

Karim, A.A., Sulebele, G.A., Azhar, M.E. and Ping, C.Y. (1999). Effect of carrageenan on yield and properties of tofu. **Food Chemistry**, v.66, n.2, p. 159-165.

Kintner, P.K. and Van Buren, J.P. (1982). Carbohydrate interference and its correction in pectin analysis using the m-hydroxydiphenil method. **Journal of Food Science**, v.47, n.3, p. 756-764.

Leonel, M. and Cereda, M.P. (2002). Physicochemical characterization of some starchy tubers. **Ciência e Tecnologia de Alimentos**, v.22, n.1, p. 65-69.

Liu, K. (1997). **Soybeans:** Chemistry, Technology and Utilization. New York: Chapman & Hall. p. 165-198.

Mahfuz, A.A., Tsukamoto, C., Kudou, S. and Ono, T. (2004). Changes of astringent sensation of soy milk during tofu curd formation. **Journal of Agricultural and Food Chemistry**, v.52, n.23, p. 7070-7074.

Min, S., Yu, Y. and Martin, S. ST. (2005). Effect of soybean varieties and growing locations on the physical and chemical properties of soymilk and tofu. **Journal of Food Science***,* v.70, n.1, p. C8-C12.

Noh, E.J., Park, S.Y., Pak, J.I., Hong, S.T. and Yun, S.E. (2005). Coagulation of soymilk and quality tofu as affected by freeze treatment of soybeans. **Food Chemistry**, v.91, n.4, p. 715-721.

Oliveira, M.A., Carrão-Panizzi, M.C., Mandarino, J.M.G., Leite, R.S., Campos Filho, P.J. and Vicentini, M.B. (2010). Quantification of the sugars, oligosaccharides and starch in special soybean genotypes/cultivars (*Glycine Max* (L) Merril) for human consumption. **Brazilian Journal of Food Technology***,* v.13, n.1, p. 23-29.

Prabhakaran, M.P., Perera, C.O. and Valiyaveettil, S. (2006). Effect of different coagulants on the isoflavone levels and physical properties of prepared firm tofu.*,* **Food Chemistry**, v.99, n.3, p. 492-499.

Seibel, N.F. and Beléia, A.D.P. (2009). Enzymatic hydrolyses of fibres from soy cotyledons and characterization of solid and soluble fractions. **Pesquisa Agropecuária Brasileira**, v.44, n.10, p. 1336-1345.

Shen, C.F., De Man, L., Buzzell, R.I. and De Man, J.M. (1991). Yield and quality of tofu as affected by soybean and soymilk characteristics: Glucono-delta-lactone coagulant. **Journal of Food Science**, v.56, n.1, p. 109-112.

Shih, M.C., Yang, K.T. and Kuo, S.J. (2002). Quality and antioxidative activity of black soybean tofu as affected by bean cultivar. **Journal of Food Science**, v.76, n.2, p. 480-484.

Somogyi, M. (1945). A new reagent for the determination of sugars. **Journal of Biological Chemistry**, n.160, n.1, p. 61-68.

Stone, H. and Sidel, J.L. (2004). **Sensory Evaluation Practices**. California: Academic Press. p. 145-244.

Swain, T. and Hillis, W.E. (1959). The phenolic constituents of *Prunus domestica* I- the quantitative analysis of phenolic constituents. **Journal of Science of Food and Agriculture**, v.10, n.1, p. 63–68.

Tang, S., Hettiarachchy, N.S., Eswaranandam, S. and Crandall, P. (2003). Protein extraction from heat-stabilized defatted rice bran: II. The role of Amylase, Celluclast, and Viscozyme. **Journal of Food Science***,* v.68, n.2, p. 471-475.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L. and Byrne, D.H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. **Journal of Food Composition and Analysis***,* v.19, n.6-7, p. 669–675.

3.2 Artigo Científico B

O artigo a seguir foi submetido ao periódico ***LWT- Food Science and Technology***.

PROTEIN EXTRACTION FROM DEFATTED SOYBEAN FLOUR WITH VISCOZYME L PRE-TREATMENT

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PROTEIN EXTRACTION FROM DEFATTED SOYBEAN FLOUR WITH VISCOZYME L PRE-TREATMENT

Michele Rosset[a], Vinicius Ricardo Acquaro Junior[b], Adelaide Del Pino Beléia

PROTEIN EXTRACTION FROM DEFATTED SOYBEAN FLOUR WITH VISCOZYME L PRE-TREATMENT

Abstract

Defatted soy flour (DSF) was pre-treated with Viscozyme L to hydrolyze cell wall polysaccharides with the objective of enhancing protein extraction. Response surface methodology (RSM) was used to study the effects of treatment variables, Viscozyme L concentration (15-45 FBG/10 g solids) and temperature (45-65 oC), on protein extraction and reducing sugars released from DSF. The regression model represented the relationship between the independent variables and the responses. Protein extraction from DSF was mainly affected by temperature, whereas the content of reducing sugars was affected by the enzyme concentration. From the RSM-generated model the optimum conditions for maximum hydrolysis of carbohydrates occurred at temperature of 45 oC with Viscozyme L concentration of 45 FBG/10 g of DSF, but the most carbohydrate hydrolysis did not result in higher protein extraction which was affected mostly by the temperature of pre-treatment, with higher extraction at the higher temperatures (55 and 65 ºC).

Keywords: Reducing sugars. Protein isolate. Response surface methodology.

1 INTRODUCTION

Soy ingredients are a good source of protein with low fat content due to their high nutritional value and low cost; soy protein is the principal commercially available vegetable protein in the world, an important alternative to proteins of animal origin. Soy ingredients are also of particular interest because they impart high functionality to many processed foods and protein isolates have the highest protein concentration among soy ingredients. Taking in consideration these advantages

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(economic, nutritive, functionality, etc.) it is important to maximize soy protein ingredients production that can contribute to a range of new food formulations. Defatted soybean flour (DSF) consists of approximately 50% protein, 40% carbohydrate and other minor components. Therefore, soy carbohydrate has the second largest concentration after protein and is a component that may influence protein extraction (Liu, 1997).

Soy protein isolates are processed from defatted soy flour using an alkaline extraction followed by isoelectric precipitation and separation by centrifugation. Yields in laboratories vary from 18% (Wang and Murphy, 1996) to 28.9% (Wang et al., 1998) and the most studied variables are pH and temperature. Barbosa, Hassimotto, Lajolo and Genovese (2006), obtained yields that varied from 27 to 35% but their main objective was to retain the most isoflavone concentration in the isolate produced.

Earlier research on enzymatic hydrolysis for protein extraction from carbohydrate rich fractions were done with various cereal sources, mostly brans, and it was determined that enzymes that hydrolyzed cell wall components increased protein extraction (Guan & Yao, 2008; Tang, Hettiarachchy, Eswaranandam, & Crandall, 2003; Ansharullah & Colin, 1997; Grossmann, Rao, & Da Silva, 1980). Seibel and Beléia (2009) used Viscozyme L to increase protein solubility from soy fiber (the solid residue of protein isolate extraction) and after hydrolysis with the carbohydrase the solubility of 76 the protein remaining in the solids increased from 7.6 to 19.5%.

Carbohydrases may have a positive effect on the extractability of plant proteins since in general they help to disintegrate the cell wall tissue and hence increase protein extraction while releasing sugars. Viscozyme L, is a multi-component carbohydrase that contains a wide range of enzymes including arabanase, cellulase, hemicellulase and xylanase (Anon, 2008), and it can effectively hydrolyze plant cell wall polysaccharides. However, pre processing of soy flour with Viscozyme L for the extraction of protein has not been previously reported.

Several factors, such as the enzyme concentration, incubation time, temperature and pH, may affect the efficiency of enzymatic treatment, and their effects may be either independent or interactive. In order to improve the efficacy of the treatment, response surface models may be developed to describe the

combination of the factors and optimization techniques can be applied to attain the optimal conditions for enzymatic treatment (Guan & Yao, 2008; Tang et al., 2003; Ansharullah & Colin, 1997; Grossmann et al., 1980).

The objective of this study was to develop an enzymatic pre-treatment using Viscozyme L to increase in the extraction of protein by hydrolysis of polysaccharides present in the defatted soy flour.

2 MATERIAL AND METHODS

2.1 Chemical And Material

Soy flour (Jasmine) was purchased in a local supermarket (Londrina, PR – Brazil). Viscozyme L (from *Aspergillus aculeatus*) was a gift from Novozymes Latin American Inc. (Araucária, PR - Brazil). The activity of Viscozyme L was Fungal Beta-Glucanase Units (FBG) g-1, in which 1 FBG is the amount of enzyme required (under standard conditions, 30 oC, pH 5.0 and 30 min reaction time) to hydrolyze barley β-glucan, to reducing power corresponding to 1 μmol glucose min-1. All the other reagents were of analytical grade.

2.2 Sample Preparation

Soy flour was defatted by shaking with hexane at 1:3 (w/v) flour-to-solvent for 1h at 25 oC. The suspension was filtered utilizing a Buchner funnel, the solids washed with more hexane, air-dried and sieved through 32 mm mesh to obtain defatted soy flour.

2.3 Enzymatic Pre-Treatment

Ten grams of DSF sample was mixed with 200 mL of deionized water at 1:20 (w/v) ratio and blended to obtain a homogeneous slurry, 15 to 45 FBG units of Viscozyme L were added and the slurries incubated in a water bath with the selected temperature (45 to 65 oC) with agitation of rpm for 30 min (variables described in 120 Table 1).

Table 1 – Levels of independents variables of the central composite routable design (CCRD).

Independent variable	Symbol		Levels				
	Uncodified	Codified	-√2	-1	0	+1	+√2
Amount of enzyme (FBG)	X_1	x_1	15	19.4	30	40.6	45
Temperature (°C)	X_2	x_2	45	48	55	62	65

2.4 Protein Extraction

Subsequent to the enzymatic treatment the slurries were adjusted to pH 9.0 with a solution of 2 molL-1 of NaOH and further incubated for 45 min at 25 oC in a shaker (rpm). The suspensions were centrifuged at 7000 *g* for 30 min at 25 oC, and the supernatants were used for protein and reducing sugars determination.

In order to evaluate the effect of enzymatic pre-treatment on protein extraction, another two independent extraction experiments, control and alkaline extraction, were performed. In the control experiment, the protein was extracted under the optimum conditions; in the alkaline method, the protein was extracted at pH 9.0 but without enzymatic pre-treatment.

2.5 Protein Determination

The protein contents of defatted soy flour, enzyme and supernatants were determined using the Kjeldahl method (AOAC, 1998) using the nitrogen conversion factor of 6.25 for the protein determination. The extracted protein was expressed as:

$$extracted\ protein\ (\%) = \frac{total\ protein\ in\ supernatant - protein\ in\ enzyme}{total\ protein\ in\ DSF}\ x\ 100$$

The proximate composition (protein, moisture, fat, ash and carbohydrate) of soy flour and enzyme were determined by the methodology of AOAC (1998).

2.6 Reducing Sugars and Glucose Determination

Extraction of reducing sugars followed the method of Leonel and Cereda (2002) and was determined by the method of Somogyi (1945) in a spectrophotometer with readings at 520 nm (UV-VIS spectrophotometer, Cintra 20) and the results were expressed in glucose equivalents (GluE). A standard curve of glucose solution was used (100 μg/mL) with a range of 10 to 100 μg. The blue color has an intensity that is proportional to the amount of reducing sugars in the sample. Glucose was determined using the Kit Glucose Bio Liquid (LABORCLIN, Brazil). The results were calculated in mg/g DSF.

2.7 Experimental Design

The 22 experimental design with a star configuration (four axial points) and four central points, totalizing 12 experiments (Table 2) was employed to study the effects of enzymatic treatments on protein extraction and reducing sugars released from DSF. The independent variables and their levels were selected based on preliminary experiments in our laboratory. The independent variables *Xi* were coded as *xi*, which are defined as dimensionless, according to the Eq. (1).

$$x_i = (X_i - X_o)/\Delta X_i \qquad \textbf{(1)}$$

where *xi* is the coded value of an independent variable, *Xi* is the real value of an independent variable, *Xo* is the real value of an independent variable at the center point, ∆*Xi* is the step change value and the response (Y values). The 12 runs were performed in a completely random order to minimize bias.

Table 2 – Central composite rotatable design (CCDR) and responsesa[a]

Run	Coded variables		Incoded variables		Response (Y)[b]		
	[FBG] x_1	Temperature x_2 (°C)	[FBG]	Temperature (°C)	Protein extracted (%)	Reducing sugars (GluE, mg/g)	Glucose (mg/g)
1	-1.0	-1.0	19.4	48	40.56	20.91	12.42
2	-1.0	1.0	19.4	62	51.84	9.50	3.88
3	1.0	-1.0	40.6	48	36.16	22.15	9.92
4	1.0	1.0	40.6	62	52.28	18.24	7.97
5	-√2	0	15	55	57.22	10.95	3.73
6	√2	0	45	55	54.68	26.00	10.03
7	0	-√2	30	45	42.12	19.57	15.13
8	0	√2	30	65	58.88	14.55	5.09
9 (C)	0	0	30	55	58.75	17.91	7.62
10 (C)	0	0	30	55	58.74	17.76	7.67
11 (C)	0	0	30	55	58.77	17.54	6.72
12 (C)	0	0	30	55	55.77	17.00	7.66

a Non-randomized.
b Averages of triplicate determination.

2.8 Statistical Analysis

The response surface regression procedure of the STATISTICA 7.0 (StatSoft, Inc., 2007) was used to fit the experimental data to the second-order polynomial equation to obtain the coefficients of Eq. 2:

$$Y = \beta_o + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \qquad (2)$$

where Y is the response variable, *xi* and *xj* are the coded independent variables, and *β*o, 180 *βi* , *βii* and *βji* are the regression coefficients of variables for the intercept, linear, quadratic and interaction regression terms, respectively. The analysis of variance (ANOVA) table was generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The regression coefficients were used for statistical calculations to generate response surfaces.

2.9 MODEL VERIFICATION

The optimum conditions of the enzymatic pre-treatment for protein extraction, enzyme concentration and incubation temperature, were obtained using RSM. For verification of the model the amount of protein extracted and reducing sugars produced under the determined optimal conditions were determined and compared to the estimated values for validation of the model.

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF THE DSF AND ENZYME

The soy flour had 38.3% protein and after defatting 47.8%, lipids were reduced from 20.5% to 3.2%. Carbohydrates calculated by difference were 29.7% in the soy 200 flour and 32.9% in the defatted flour. The enzyme had 7.9% protein (Table 3).

Table 3 – Proximate composition (%) of Viscozyme L, soy flour and defatted soy flour[a]

Component	Viscozyme L	Soy flour	Deffated soy flour
Protein	7.95 ± 0.15	38.28 ± 0.01	47.79 ± 0,15
Moisture	----	7.17 ± 0.04	11.02 ± 0.09
Ash	----	4.36 ± 0.05	5.07 ± 0.02
Fat	----	20.52 ± 0.03	3.17 ± 0.22
Carbohydrate[b]	----	29.67	32.95

a Values represent the means of three determinations ± standard.
b Calculated by difference.

3.2 Model Fitting

The independent and dependent variables were analyzed to obtain a regression equation that could estimate the response within the studied range for temperature and enzyme concentration. The determination of total reducing sugars, as glucose equivalents (GluE) was to follow carbohydrate hydrolysis during the pre-treatment to determine whether it affected protein extraction. For carbohydrate hydrolysis estimated as an increase in glucose equivalents the linear term of the enzyme concentration was the most important variable and as the enzyme concentration increased hydrolysis of carbohydrates increased. The quadratic term of temperature was incorporated into the lack of fit for calculation of the R-squared and F-ratio because they had no significant effect on the responses. The regression equations for glucose equivalents released and protein extracted (Y) are as follows,

Reducing sugars (GluE, mg/g of DSF) =

$$17.3 + 3.9x_1 + 0.5\, x_1^2 - 2.8x_2 - 1.9\, x_1x_2 \qquad \textbf{(3)}$$

Protein extracted (%) =

$$58.1 - 0.9\, x_1 - 3.0\, x_1^2 + 6.4x_2 - 5.7\, x_2^2 + 1.2\, x_1x_2 \quad \textbf{(4)}$$

where, $x1$ is enzyme concentration and $x2$ is temperature.

Unlike the model proposed for glucose content, analysis of the effects indicated that the amount of extracted protein was mostly influenced by temperature of the pre-treatment. There was no interaction between the enzyme concentration and incubation temperature.

The plot of experimental values of reducing sugars (GluE, mg/g) and protein extracted versus those calculated from Eq. (3) and Eq. (4), respectively, indicated a good fit (plots not shown). For the fitted model the coefficients of determination ($R2$) for total reducing sugars release and protein extracted, which is a measure of degree of fit, were 0.887 and 0.807, respectively. This implies that the

enzyme concentration and the temperature could explain 88.77 and 80.77% of the variations. Joglekar and May (1987) suggested that, for a good fit of a model, *R2* should be at least 0.80. Therefore, the developed model could adequately represent the relationship among the parameters chosen. The results of analysis of variance for the CCRD and the significance of each coefficient determined by the *F*-test are shown in Table 4; the corresponding variables would be more significant if the absolute *F*-value calculated is greater than *F*-value tabulated.

Tabela 4 – Analysis of variance (ANOVA) of the regression parameters for the response surface model.

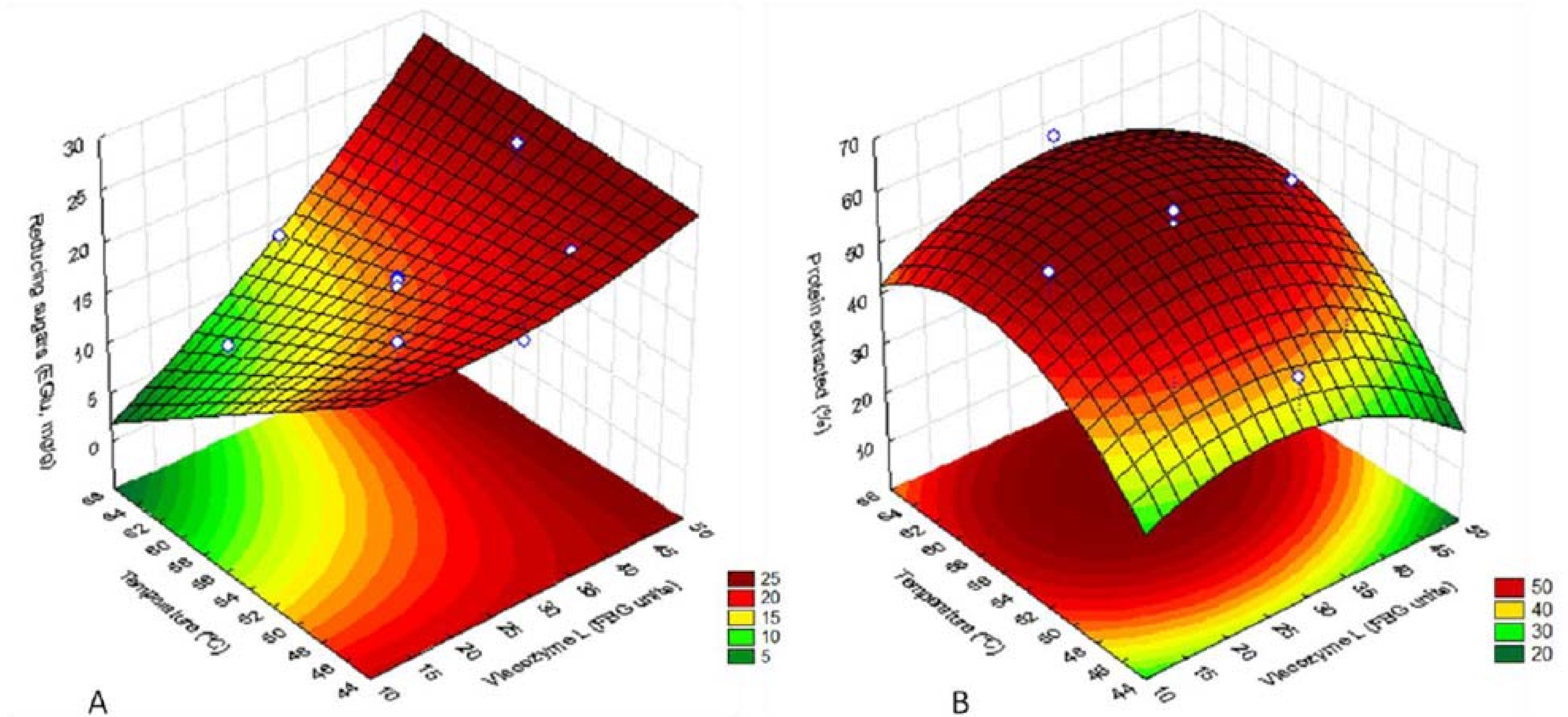
Regression	Sum of square	Degree of freedom	Mean square	*F* Value calc	*F* Value tab
Reducing sugars (EGlu, mg/g)[a]					
Regression	200.6163	4	50.1541	13.8387	4.1203
Linear effects	184.9666	2	92.4833	25.5182	4.7374
Quadratic effects	1.6397	1	1.6397	0.4524	5.5914
Interaction effects	14.0100	1	14.0100	3.8657	5.5914
Error	25.3693	7	3.6242		
Total SS	225.9856	11			
% Protein extraction[b]					
Regression	610.4225	5	122.0845	5.3514	4.3874
Linear effects	333.5589	2	166.7795	7.3105	5.1433
Quadratic effects	271.0072	2	135.5036	5.9396	5.1433
Interaction effects	5.8564	1	5.8564	0.2567	5.9874
Error	136.8823	6	22.8137		
Total SS	747.3048	11			

a Regression coefficient: R = 0.88774; Adjusted coefficient: R = 0.82359
b Regression coefficient: R = 0.80776; Adjusted coefficient: R = 0.64756

The coded models expressed by Eq. (3) and (4) were used to generate the response surfaces for reducing sugars in glucose equivalents (Figure 1A) and protein extracted in the supernatant, respectively (Figure 1B). The results indicated that enzyme concentration displayed a linear effect on the response, and that total reducing sugars content increased with an increase in enzyme concentration with temperature demonstrating a negative linear effect on the

response. The results indicated that temperature exerted linear positive and quadratic negative effect on response, whereas, the effect on enzyme concentration was quadratic negative. For protein extraction temperature of pre-treatment had the most effect, with maximum extraction at temperature between 55 and 65 oC.

Figure 1 – Response surface for the effects of enzyme concentration and temperature on reducing sugars released (A) and protein extracted (B) from DSF.



3.3 Optimum Conditions and Model Verification

From de model, optimum conditions for enzymatic pre-treatment of DSF for carbohydrate hydrolysis were obtained (Table 5). Under the optimum conditions of enzyme concentration 45 FBG at 45 oC, a maximum response of 24.1 mg/g of DSF was estimated. The experimental value of 25.3 mg/g of DSF was found with an error of 5%.

From de model, optimum conditions for enzymatic pre-treatment of DSF for protein extraction were obtained (Table 5). Under the optimum conditions, 30 FBG and 60 oC the predicted protein extracted was 59.6%, and the determined value was 56.3% with an error of 5.7%. Those results indicated that the generated regression model represented the relationship between the independent variables and the responses.

Table 5 – Optimum conditions of enzymatic treatment, predicted and experimental results of reducing sugars (GluE) and protein values from RSM.

	Optimum condition		Responses	
	[enzyme] (FBG)	Temperature (°C)	Predicted value	Experimental value[a]
Reducing sugars (GluE, mg/g)	45	45	24.08	25.28 ± 1.21
Protein extracted (%)	30	60	59.65	56.27 ± 0.46

a Means ± standard deviation of triplicate determinations.

Guan and Yao (2008), using 30 FBG of Viscozyme L/10 g of defatted oat bran found that under the optimum conditions of 44 oC, pH 4.8 and incubation time of 2.8 h, the predicted protein extraction was 55.7% and the experimental extracted protein was 56.2%. Tang et al. (2003) verified the effects of amylase, Viscozyme L, and Celluclast on protein extraction from heat-stabilized defatted rice bran. The maximum protein extracted for amylase, Viscozyme (80 FBG/10 g sample), and Celluclast was 45.4, 28.5, and 12.1%, respectively, under optimal pH and temperature, and 10:1 water to bran ratio. Seibel and Beléia (2009) increased protein extraction (62%) from soybeans cotyledon fibers using Viscozyme L, but used

a higher enzyme concentration (20 FBG/g of sample) in a substrate that contained 65% total carbohydrates.

The use of the enzyme at the optimum conditions, with main the effect being the temperature of the pre-treatment, resulted in an increase of 70.3% of proteins extracted compared alkaline method (Table 6). Grossmann et al. (1980) reported that proteins from buckwheat bran were extracted by alkaline extraction process, giving a yield of 37.0% of the total nitrogen in the liquid supernatant, at pH 6.5. However, the yield could be improved, given a pre-treatment of the bran with carbohydrases, thus producing a yield of 56% with a cellulase, 58% with a pectinase, whereas the increase was minimal with hemicellulase. The maximum yield of 67.5% was obtained by synergistic action of pectinase and hemicellulase, in a treatment lasting for 7 h at pH of 3.7.

Table 6 – The effects of Viscozyme L pre-treatment on DSF protein extraction.

Treatments	Extracted protein (%)[a]
Enzymatic treatment method	56.27 ± 0.46
Alkaline method[b]	33.04 ± 0.72

a Means ± standard deviation of triplicate determinations.
b With alkaline solution at pH 9.0.

3.4 Effect of Independent Variables on Protein Response

Protein extracted varied from 36.2% with 41 FBG and 48 ºC to 58.9% using 30 FBG and 65 ºC with the linear and quadratic effects of the temperature being the most important variable. For protein extraction, the central point with 30 FBG and 55 ºC released the same amount of protein, an average of 58%, as the amount released with 30 FBG and 65 ºC. Analyzing treatments 7 and 8, with the same enzyme concentrations (30 FBG), and temperatures of 45 and 65 oC, respectively (Table 2), there was an increase of 39.8% of protein extracted in treatment 8. It is important to notice that the pre-treatment temperature influenced the amount of protein extracted although the extraction occurred at 25 °C.

Guan and Yao (2008) verified that protein extraction, after pre-treatment with Viscozyme L of oat bran, was influenced mostly by temperature and pH of the extraction, but the amount of enzyme had a linear significant effect. Wang and Murphy (1996) obtained 21% protein from DSF with an alkaline extraction at 25 °C. Rickert, Meyer, Hu, & Murphy (2004) extracted 63% of protein from DSF at 60 °C and pH 8.5, and 69% of protein at 60 °C and pH 10.5, demonstrating the influence of temperature on protein extraction. Ansharullah and Colin (1997) verified that in higher temperature the protein extraction (measured as nitrogen extracted) was more efficient. They observed that at 40 oC, pre-treatment with Viscozyme L and Celluclast 1.5L, the nitrogen extracted from rice bran was 51%, whereas at 50 oC, and Viscozyme L, it was 57%, control treatment extracted 25.75 of the total N, but they used a higher concentration of enzyme (120 FBG/10g of bran). Seibel and Beléia (2009) increased protein solubility from soy fiber used 20 FBG/ g of sample.

3.5 Effect of Independent Variables on Carbohydrates

Total reducing sugars varied between 9.5 to 26 mg/g of DSF and the linear variability of the enzyme concentration was the most important independent variable, with the maximum hydrolysis with 45 FBG units. In treatment 2, temperature of 62 oC, total reducing sugar concentration was lower than in treatment 5, where the applied temperature was 55 oC (Table 2). That is, the effect of enzyme concentration is greater than the effect of temperature and temperatures above 60 oC are not recommended since it is very close to the inactivation temperature in accordance to Novozyme, (optimum temperature range from 25 to 55 oC).

The total cell wall polysaccharides of fourteen soybean varieties represented on average 21% of the grain, and major components were galactose, glucose, arabinose and uronic acids, whereas xylose, rhamnose and fucose were found in smaller quantities (Huisman, Schols, & Voragen, 1998; Stombaugh, Jung, Orf, & Somers, 2000). The multi-component nature of Viscozyme L, which contained a wide range of carbohydrases including arabinase, cellulase, hemicellulase and xylanase (Anon, 2008), seemed to be advantageous in cleaving the linkages within the polysaccharide matrix, and hence liberating more intercellular constituents like

protein, but Viscozyme L had no significant effect in increasing the extractability of protein from DSF.

Glucose concentration varied in a different way with a minimum of 3.7 mg/ g of DSF (34% of the total reducing sugars) and a maximum of 15.1 mg/g of DSF where it represented 77.3% of the reducing sugars produced using 30 FBG at 45 ºC (Table 2). Ouhida, Pérez and Gasa (2002) identified monosaccharide hydrolyzed from soybean cell wall and galactose had the highest concentration, followed by glucose, arabinose and uronic acids, with main polysaccharides being cellulose, xyloglucan and pectic substances. Hanmoungjai, Pyle and Niranjan (2002) reported that Viscozyme L was a very effective enzyme in hydrolyzing carbohydrates and releasing reducing sugars, but did not improve yields of oil or protein from rice bran.

CONCLUSION

Carbohydrate hydrolysis as measured as GluE occurred in the DSF but Viscozyme L pre-treatment could not improve the protein extraction from DSF in the range of enzyme concentration studied. The highest temperature used in the pre-treatment resulted in the most protein extraction while enzyme concentration had no effect.

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REFERENCES

Anon (2008). **Product sheet of Viscozyme L.** Novo Nordisk A/S, Enzymes process division, Bagsvaerd, Denmark.

Ansharullah, J. A. H., & Colin, F. C. (1997). Application of carbohydrases in extracting protein from rice bran. **Journal of the Science of Food and Agriculture**, v.74, n.2, p. 141-146.

AOAC (1998). **Official Methods of Analysis**, (20th ed.). Arlington: Association of Official Analytical Chemists.

Barbosa, A. C. L., Hassimotto, N. M. A., Lajolo, F. M., & Genovese, M. I. (2006). Teores de isoflavonas e capacidade antioxidanteda soja e produtos derivados. **Ciência e Tecnologia de Alimentos**, v.26, n.4, 921-926.

Grossmann, M. V., Rao, C. S., & Da Silva, R. S. F. (1980). Extraction of protein from buckwheat bran: Application of enzymes. **Journal of Food Biochemistry**, v.4, n.3, p. 181-188.

Guan, X., & Yao, H. (2008). Optimization of Viscozyme L- assisted extraction of oat bran protein using response surface methodology. **Food Chemistry**, v.106, n.1, p. 345-351.

Hanmoungjai, P., Pyle, D. L., & Niranjan, K. (2002). Enzyme-assisted water-extraction of oil and protein from rice bran. **Journal of Chemical Technology and Biotechnology**, v.77, n.7, p. 771-776.

Huisman, M. M. H., Schols, H. A., & Voragen, A. G. J. (1998). Cell wall polysaccharides from soybean (*Glycine max*.) meal. Isolation and characterization. **Carbohydrate Polymers**, v.37, n.1, p. 87–95.

Joglekar, A. M., & May, A. T. (1987). Product excellence through design of experiments. **Cereal Foods World**, v.32, n.12, p. 857–868.

Leonel, M., & Cereda, M. P. (2002). Physicochemical characterization of some starchy tubers. **Ciência e Tecnologia de Alimentos**, v.22, n.1, p. 65-69.

Liu, K. (1997). **Soybeans:** Chemistry, Technology and Utilization. New York: Chapman & Hall.

Ouhida, I., Pérez, J. F., & Gasa, J. (2002). Soybean (*Glycine max.*) cell wall composition and availability to feed enzymes. **Journal of Agricultural and Food Chemistry**,v.50, n.7, p. 1933-1938.

Rickert, D. A., Meyer, M. A., Hu, J., & Murphy, P. A. (2004). Effect of extraction ph and temperature on isoflavone and saponin partitioning and profile during soy protein isolate production. **Journal of Food Science**, v.69, n.8, p. 623-631.

Seibel, N. F., & Beléia, A. D. P. (2009). Enzymatic hydrolyses of fibers from soy cotyledons and characterization of solid and soluble fractions. **Pesquisa Agropecuária Brasileira**, v.44, n.10, p. 1336-1345.

Somogyi, M. (1945). A new reagent for the determination of sugars. **Journal of Biological Chemistry**, v.160, n.1, p.61-68.

StatSoft. (2007). **Statistica for Windows**, Tulsa: Stasoft.

Stombaugh, S. K., Jung, H. G., Orf, J. H., & Somers, D. A. (2002). Genotypic and environmental variation in soybean seed cell wall polysaccharides. **Crop Science,** v.40, n.2, 408-412, 2000.

Tang, S., Hettiarachchy, N. S., Eswaranandam, S., & Crandall, P. (2003). Protein extraction from heat-stabilized defatted rice bran: II. The role of Amylase, Celluclast, and Viscozyme. **Journal of Food Science***,* v.68, n.2, p. 471-475.

Wang, C., Ma, Q., Pagadala, S., Sherrard, M. S., & Krishnan, P. G. (1998). Changes of isoflavones during processing of soy protein isolates. **Journal of the American Oil Chemists' Society***,* v.75, n.3, 337-341.

Wang, H. J., & Murphy, P. A. (1996). Mass balance study of isoflavones during soybean processing. **Journal of Agricultural and Food Chemistry***,* v.44, n.8, p. 2377-2383.

3.3 ARTIGO CIENTÍFICO C

O artigo a seguir será submetido ao periódico ***Food Chemistry***.

PRE-TREATMENT OF SOY SLURRY WITH VISCOZYME L AND THE CONCENTRATION OF SUGARS AND ISOFLAVONES AND THE MICROSTRUCTURE OF SILKEN TOFU

Michele Rosset, Adelaide Del Pino Beléia

PRE-TREATMENT OF SOY SLURRY WITH VISCOZYME L AND THE CONCENTRATION OF SUGARS AND ISOFLAVONES AND THE MICROSTRUCTURE OF SILKEN TOFU

Michele Rosset[1], Adelaide Del Pino Beléia[2]

Abstract
Silken tofu which retains all solids present in the soymilk was investigated for differences in the microstructure, sugars and isoflavones content. Enzymatic pre-treatment of soy slurry was carried out with FBG/10 g solids at 55 ^{o}C, for 30 minutes. Stachyose was the predominant oligosaccharide in the treated tofu, 4.58 g/100 g and 3.30 g/100 g in control tofu, raffinose content was 1.21 and 0.75 g/100 g in treated and control tofu, respectively. Glucose was approximately duplicated in treated tofu (1.66 g/100 g) compared to control (0.74 g/100 g) while fructose content was three times higher (1.09 g/100 g). Comparing isoflavones content, malonyl conjugates were double the amount of the control tofu, with the exception of malonylgenistin, but the total amount of isoflavones was not different. The tofu samples had a globular microstructure of protein that was integrated into clumps and showed a fibrous three-dimensional network structure. The tofu of the treated slurry was organized with a well-defined three-dimensional honeycomb-like network, but with larger protein aggregates and was more tightly connected than the control.

Keywords: Carbohydrase. Oligosaccharides. Soybean curd. Scanning electron microscopy.

1 INTRODUCTION

Soybean is one of the major food sources worldwide providing proteins, oil, carbohydrates, bioactive compounds like isoflavones, and many other nutrients to humans and animals. Soybean contains approximately 40% protein, 20% oil, and 33% carbohydrates and up to 16.6% of the carbohydrates are soluble sugars or oligosaccharides. Glucose, fructose, sucrose, raffinose and stachyose comprise almost 99% of the soybean soluble sugars (Hymowitz, Collins, Panczner, & Walker, 1972).

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Twenty eight Brazilian cultivars analysed in 2010 had concentration of stachyose that varied from 2.69 to 3.69 g/100 g, raffinose from 0.48 to 1.19 g/100 g and sucrose from 3.25 to 5.95 g/100 g. In this same report BRS 267 had 2.23% of stachyose, 0.94% of raffinose and 5.95% of sucrose. The use of cultivar BRS 267 can improve the sensory quality of soy products like tofu and soymilk due to the high sucrose content and could mask astringency, beany flavor and other flavorful compounds such as saponins and isoflavones (Oliveira et al., 2010).

Sugars affect soy food quality and nutritional values. Soluble sugars, especially sucrose, are the major source of energy for fermentation and contribute to the sweetness of soymilk and tofu. Increased total sugar and sucrose content is important to achieve the proper rate of fermentation during the natto-making process and to improve the taste and flavor of natto and tofu (Taira, 1990).

Although oligosaccharides, including raffinose and stachyose, are considered undesirable sugars in soybean, because they are not readily digestible and cause flatulence or diarrhoea, they can act as prebiotcs or as a source of soluble dietary fiber (Mussatto, S. I., & Mancilha, 2007).

In addition, soybeans contain others health benefiting phytochemicals of which isoflavones have been reported to be the most important. Numerous reports have indicated that isoflavones may possess the biological activities to inhibit menopause symptoms in women, cancer cell proliferation and oxidative stress (Coward, Barnes, Setchell, & Barnes, 1993; Bedani & Rossi, 2005; Martínez, 2006). There are 12 chemical forms of isoflavones in soybeans and soy foods, consisting of 3 chemical forms that are aglycons and the 9 conjugated compounds as β-glucoside, malonyl-β- glucoside, acetyl-β-glucoside (Liu, 1997). It has been established that their chemical form in foods can influence the bioavailability of isoflavones and that the compounds may be altered since they are susceptibility to degradation and alteration especially during heating (Coward, Smith, Kirk, & Barnes, 1998).

The concentrations of each of these forms will vary in soy foods depending upon the type of processing. In soy foods that are processes with water, the native soy β-glucosidases will be active prior to any heat treatment generating aglycons as it happens during the soaking of soybeans prior to processing into soymilk. Heat treatment of moist soy foods, as in soymilk and tofu, will tend to

generate β-glucosides at the expense of the malonyl forms. Tofu is a traditional oriental soybean food made by the coagulation of soluble proteins with a calcium or magnesium salt or glucono-δ-lactone (GDL), from hot-water extract of whole soybeans. Glucono-δ-lactone, is an acid precursor used as a protein coagulant especially in making silken‖ that have a shelf-life is greatly extended when compared to soft tofu. Without removing the whey from the curd, GDL-coagulated silken tofu has a custard-like texture and smooth mouth feel (Liu, 1997).

Treatment of protein with proteases is common, but less studied is the hydrolysis of carbohydrates in soybeans by carbohydrases. The addition of the carbohydrase in soy slurry before obtaining the soymilk may result in tofu with technological properties and composition different from the traditional tofu, especially in the concentration of sugars to increase the sweetness of the product. The use of a multienzymatic complex, such as Viscozyme L, which contains cellulases, arabinases, hemicellulases, gluconases and xylanases (Anon, 2008), may cause the hydrolysis of the carbohydrates in cell walls, favoring the extraction of useful compounds (protein and sugars) from the vegetable tissues. Silken tofu product was chosen because the products of the hydrolysis are water-soluble and in a soft tofu (pressed) these sugars would be eliminated in the whey.

The presence of low molecular weight co-solutes such as sugars in the aqueous phase of food systems can alter the conformation and interactions of proteins by binding to protein surface groups (Gu, Campbell, & Euston, 2009). The objectives of this study were to verify the influence of Viscozyme L pre-treatment of soy slurry on sugars and isoflavones content in treated tofu compared to control and to analyze the microstructure of these different products.

2 MATERIAL AND METHODS

2.1 Chemical And Material

The multi-enzymatic complex Viscozyme L (from *Aspergillus aculeatus*) was a gift from Novozymes Latin American Inc. (Araucária, PR- Brazil). The activity of Viscozyme L was 100 Fungal Beta-Glucanase Units (FBG) g-1, in which 1 FBG is the amount of enzyme required under the standard conditions (30

oC, pH 5.0 and 30 min reaction time) that hydrolyzes barley β-glucan to reducing carbohydrates, corresponding to 1 μmol glucose min-1.

The soybean variety BRS 267 (large grains), crop 2007/2008 (EMBRAPA – PR, Brazil), was used to prepare the tofus. The antifoam agent was supplied by Prosabor (São Paulo-Brazil) and GDL (Sigma-Aldrich) was used as coagulant agent. All the other reagents were of analytical grade.

2.2 Production of Silken Tofu

The tofus were prepared by a modification of the methods proposed by Min, Yu, and Martin (2005) and Shen, De Man, Buzzell, and De Man (1991). Soybeans (approximately 150 g) were soaked in 1500 ml distilled water at room temperature for 16 h. The soaked soybeans were drained, rinsed and ground with 1000 mL distilled hot water (98 oC) in a blender for 3 min at high speed. Antifoam agent (1.0 mL) was added to the slurry during grinding.

Viscozyme L (2.5 mL, 30 FBG/10 g solids) was added to 1000 mL portion of soy slurry. After incubation at 55 oC for 30 min, the soy suspension was boiled (98 oC, 10 min) and filtered manually with a muslin cloth to obtain soymilk. To a 400 mL portion of soymilk (45 oC), 10 mL of GDL (0.02 M) was added and placed into a plastic container which was transferred to a water bath (Marconi, MA 127) and left at 85 oC for 60 min. The yield of tofu was calculated as the weight of fresh tofu obtained from a specified amount of the soymilk (400 mL) used for its preparation. Tofus without the enzyme treatment (control) were produced in the same way, excluding only the step of enzyme addition. The tofus were frozen and lyophilized (0.040 mbar and -50 oC) in a freeze-dryer (Alpha 2-4 LD Plus, Christ) to determine sugars and isoflavones. The moisture content of lyophilized tofu with enzyme and control was 3.6% and 2.8%, respectively.

2.3 Extraction And Quantification of Soluble Sugars From Tofus

The extraction of sugars (glucose, fructose and sucrose) and oligosaccharides (raffinose and stachyose) followed the method of Masuda, Kaneko, and Yamashita (1996) adapted by Mandarino, Carrão-Panizzi, and Masuda (2000)

with some modifications. Approximately, 1.0 g of freeze dried tofu was suspended in 10 mL of 80% ethanol, and the dispersion was homogenized for 2 min, with the aid of a Polytron. The samples were centrifuged (Sorvall TC 6, Du Pont) at 2000 x *g* for 5 min, the supernatant was decanted and to the precipitate another 10 mL of 80% ethanol was added for a second extraction. The supernatants were combined in a 50 mL centrifuge tube and the volume was completed to 25 mL. The content of the tube was homogenised, and 100 µL of the clear supernatant was mixed with 1.4 mL of a 80% ethanol solution.

Sugars and oligosaccharides were quantified by high performance liquid chromatography (HPLC) in a Dionex Bio LC chromatograph, equipped with automated sampler (TSP, model Spectra System AS 3500) and electrochemical detector with gold electrode (Dionex model ED 50). Sugars were separated on a Dionex CarboPac PA 10 anion-exchange resin column (250 mm x 4 mm i.d.), preceded by a Dionex CarboPac PA 10 guard column (50 mm x 4 mm i.d.). The mobile phase consisted of 50 mM NaOH solution prepared by dilution of carbonate-free 50% (w/w) NaOH solution in MilliQ water, with a flow rate of 1.2 mL/min at approximately 25 °C.

HPLC identification and quantification of the major sugars present in the samples was achieved by comparing each peak retention time and area with those of external standards. A stock solution composed of glucose, fructose, sucrose, raffinose, and stachyose with concentrations of 40, 80, 120, 160, and 200 ηmoles was prepared for the HPLC system. A standard curve for each sugar was prepared by injecting different concentrations of the stock solution. All sugar analyses were performed in triplicate and results were expressed on a dry-weight basis in g/100 g.

2.4 Isoflavone Extraction And Quantification by HPLC

The quantitative analysis of isoflavones was conducted according Kudou et al. (1991) adapted by Carrão-Panizzi and Bordingnon (2000). The tofu samples were freeze-dried and defatted, and these dried samples were used in the isoflavone analyses. Extracting the oil from soybeans or its products does not remove the isoflavones since they are not soluble in hexane. Isoflavones were extracted from 100 mg samples of defatted freeze-dried tofus. Samples were placed in test tubes

with 4 ml of 70% aqueous ethanol containing 0.1% acetic acid, at room temperature and extracted for 1 hour with agitation at 15 min intervals. Then, the tubes were sonicated for 30 minutes and the extract (approximately 1.5 mL) were transferred to an Eppendorf tube and centrifuged at 21000 x *g* for 10 min at 5 oC (Centrifuge Eppendorf, model 5417R). After centrifugation, the supernatants were filtered into vial through Millex 0.45 µm using a syringe filter (Arti Glass SRL, Italy) and transferred to the auto sampler (20 µl was the volume injected). Analysis of isoflavones was performed with a reverse-phase column (CLC-ODS (M) C18, 4.6 mm internal diameter x 250 mm, 5 mm particles). The mobile phase (solvent A) was a solution of acetonitrile and 0.1% acetic acid, while solvent B was a solution of water and 0.1% acetic acid. Initial gradient was 20% for the solvent A in the first 20 minutes, passing to 100% for 5 minutes and 20% for the last 15 minutes. The isoflavonas were detected at 260 nm. The complete elution of each sample was performed in 40 minutes. Standard solutions of daidzin, daidzein, genistin and genistein (Sigma-Aldrich) were 0.0125 mg/ml. The isoflavone contents were expressed in mg/100 g of tofu on a dry weight basis.

2.5 Scanning Electron Microscopic Observation

The procedure used for sample preparation was that of Kao, Su, and Lee (2003) with some modifications. A small piece of each tofu sample was pre-fixed at room temperature with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h. The samples were freeze-fractured in liquid nitrogen and washed (three times) with 0.1 M phosphate buffer (pH 7.0) at 15 min intervals, and were then post-fixed with 1% osmium tetroxide in the same buffer for 60 min at room temperature. The fixed samples were again rinsed three times with phosphate buffer at 15 min intervals. Then the samples were dehydrated in a graded series of ethanol solutions (70%, 80%, 90% and 100%). The samples in alcohol were placed into a vacuum evaporator to sublimate the ethanol (Critical Point Dryer CPD 010, Bal-Tec). After sublimation, the dried samples were fixed in the microscope stub by using double-sided adhesive carbon-tapes. The broken side of each cubic sample faced the top of the aluminium stub and was sputter-coated with gold (Sputter Coater model SCD

050, Bal-Tec). The observations were carried out at 20 kV by a scanning electron microscope (model Quanta 200, FEI).

2.6 Statistical Analyses

All extractions and determinations were performed in triplicate. Results are expressed on a dry matter basis as mean values ± standard deviation. Analysis of variance (ANOVA) was conducted for the data collected, using Statistica 7.0 for Windows (StatSoft Inc., USA). Differences among the mean values were determined using Student t-test. A significant level was defined as a probability of 0.05.

3 RESULTS AND DISCUSSION

3.1 Sugars and Oligosaccharides

A HPLC chromatogram of the sugars from control tofu and treated tofu is show in Figure 1 (A and B, respectively). Total sugars were 11.69 in treated tofu and 8.93 g/100g in control sample, a 30.9% increase (Table1). Control tofu had as major sugar sucrose 3.77 g/100g, followed by stachyose 3.30 g/100g, with fructose being the minor component, 0.36 g/100g. The treated sample had 4.58 g/100g of stachyose, the predominant oligosaccharide, followed by 3.08 g/100g of sucrose, but with a higher concentration of fructose and glucose compared to the control sample. Glucose had twice the concentration, 1.66 g/100g and fructose three times more than control with 1.09 g/100g. During silken tofu manufacturing, there is no removal of whey like in soft tofu, so soluble sugars present in the soymilk will remain in the tofu. Treated tofu could have a sweeter taste (more glucose and fructose) and had more oligosaccharides (raffinose and stachyose) than control tofu.

Soybean oligosaccharides, raffinose and stachyose, which resist digestion due to the α-galactoside linkages in their structure have prebiotic effects, and studies have shown that their consumption is related to several health benefits, such as lowering blood cholesterol, reducing blood pressure and preventing some

types of cancer (Mussatto & Mancilha, 2007). The treated tofu had a total of 5.79 and control had 4.05 g/100g of oligosaccharides, an increase of 43%.

Considering the sugars determined in the Brazilian cultivar BRS 297 in 2010, 150 g of soy used for tofu production had a calculated amount of 8.92 g of sucrose and 3.34 g of stachyose, of which 3.56 and 1.33 would be in the 400 mL of soy milk used in the tofu, but these values were present in defatted soy flour and would be less in the soybean grain. The amounts found in the tofus were higher for stachyose and sucrose as calculated, but the amount really present in the grain was not determined.

Figure 1 – Quantification of sugars in control (A) and treated tofu (B). Glucose (1), fructose (2), sucrose (3), raffinose (4) and stachyose (5).

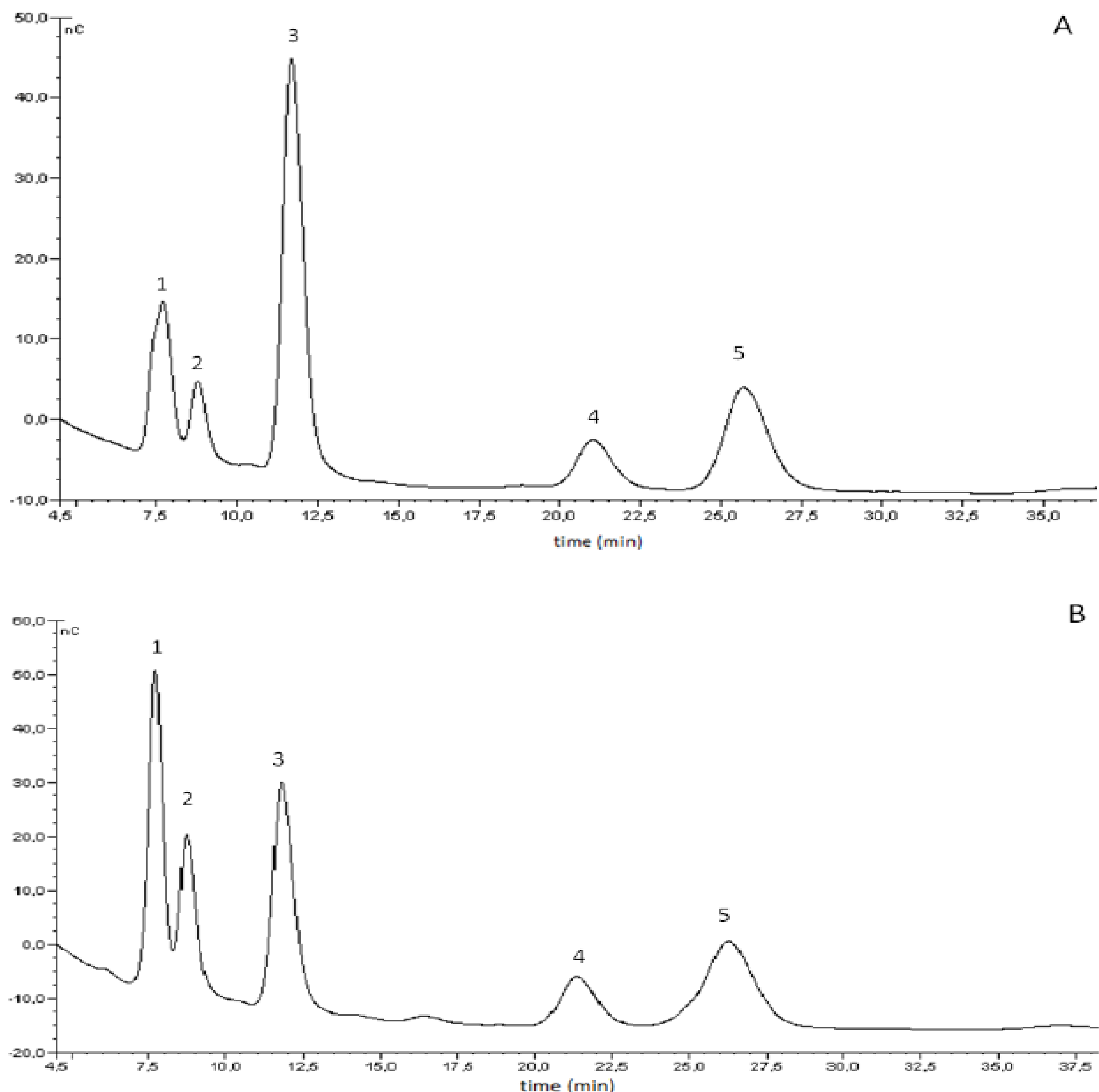


Table 1 – Sugar composition (g/100 g, db) of tofus.

Sugar	Control tofu	Treated tofu
Glucose	0.74 ± 0.02 [b]	1.66 ± 0.08 [a]
Fructose	0.36 ± 0.02 [b]	1.09 ± 0.03 [a]
Sucrose	3.77 ± 0.26 [a]	3.08 ± 0.03 [a]
Raffinose	0.75± 0.01 [b]	1.21 ± 0.04 [a]
Stachyose	3.30 ± 0.19 [b]	4.58 ± 0.16 [a]
Total sugars	8.93 ± 0.50 [b]	11.69 ± 0.34 [a]

Means values in the same row followed by different letters are different (Student *t*-test, $p \leq 0.05$).

3.2 Isoflavones

A HPLC chromatogram of the isoflavones extracted from control tofu and treated tofu is show in Figure 2 (A and B, respectively). Aglycons with the exception of genistein, which was lower in treated tofu, had equal concentrations. β-glucosides were lower in treated tofus than in control, since the carbohydrases could have de-esterified the glucose molecule. The levels of malonyl conjugates were approximately double compared to control (Table 2). Apparently the amount of malonyl forms initially present in the soybean grain were less altered in the treated tofu while the β-glucosides had a higher level of hydrolysis. The concentrations found of the various forms of isoflavones in the control tofu were very similar to the values reported by Wang and Murphy (1996).

Figure 2 – A HPLC profile of isoflavones extracted from control tofu (A) and treated tofu (B). The peaks are identified as 1. Daidzin, 2. Glycitin, 3. Genistin, 4. Malonyldaidzin, 5. Malonylglycitin, 6. Malonylgenistin, 7. Daidzein, 8. Glycitein, 9. Genistein**.**

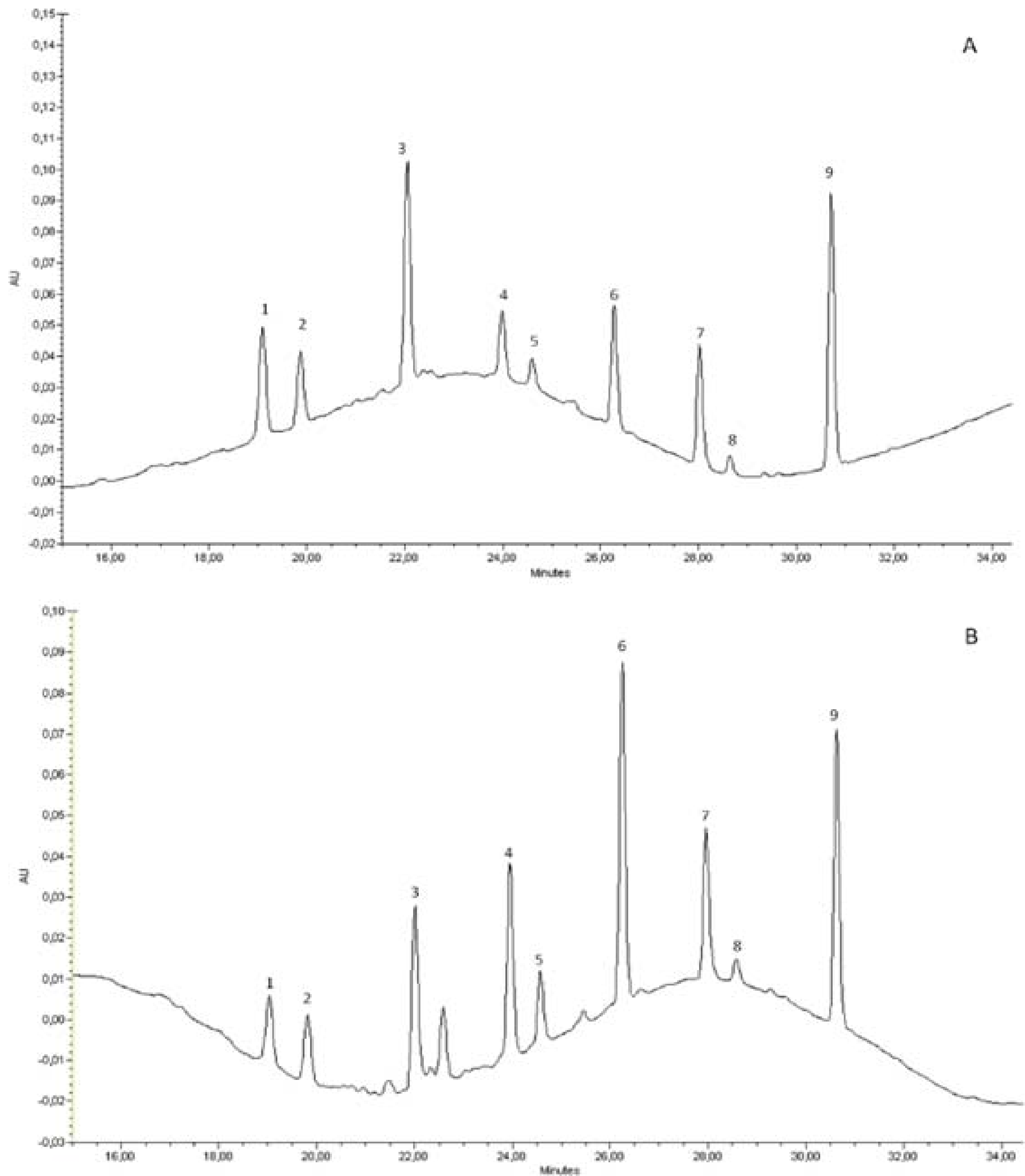


Table 2 – Isoflavones composition (mg/100 g, db) of tofus.

Isoflavone	Control tofu	Treated tofu
Daidzein	10.44 ± 0.77 [a]	9.85 ± 0.93 [a]
Glycitein	2.93 ± 0.16 [a]	2.89 ± 0.09 [a]
Genistein	19.94 ± 0.03 [a]	16.14 ± 0.11 [b]
Daidzin	22.20 ± 0.63 [a]	10.92 ± 0.46 [b]
Glycitin	14.24 ± 0.27 [a]	10.14 ± 0.57 [b]
Genistin	28.90 ± 1.75 [a]	18.15 ± 1.39 [b]
Malonyldaidzin	11.65 ± 0.12 [b]	26.58 ± 0.59 [a]
Malonylglycitin	7.91 ± 1.01 [b]	15.65 ± 1.61 [a]
Malonylgenistin	15.32 ± 0.45 [b]	32.77 ± 0.19 [a]

Means values in the same row followed by different letters are different (Student *t*-test, $p \leq 0.05$).

Soybean grains had the majority of the compounds in the malonyl and β-glucosides forms with very low amounts of aglycon forms and in the produced tofu there was an increased amount of aglycons (Wang and Murphy, 1996) who found 7.2, 13.2 and 5.5 mg/100 g while we found 9.85, 16.14 and 2.89 mg/100g for daidzein, genistein an glycitein and no difference with the concentration in control tofu.

Hui, Henning, Park, Heber, & Go (2001) determined the variation of the isoflavone content of 23 commercially available packaged tofus by brands and batches. Among different tofu brands the genistein and daidzein/glycitein content varied from 0.38 to 1.47 mg/100 g and 0.39 to 1.14 mg/100 g dry basis, respectively. The isoflavone content from batch to batch of the same brand varied between 7 and 28% based on dry weight. According Hui et al. (2001), assuming a daily consumption of 30 g commercial tofus products, the isoflavone intake could vary between 2.1 to 13.2 mg/day, with higher levels in pressed tofus. Considering our tofus, with approximately 91% of water, the intake could vary between 3.6 and 3.9 mg/day for control and enzyme treated tofu, respectively.

Fukutake et al. (1996) reported lower amounts of genistein e genistin in tofu (94.8 to137.7 μg/g), probably tofus produced by pressing the curd when the

isoflavones were probably liberated with the whey, since they are water soluble. So there is an advantage in producing silken tofu to obtain more isoflavones compared to other types of tofus. In non-fermented soy products, isoflavones are present mainly as β-glucoside conjugates like we found in the control tofu (Table 2).

From the grain to the tofu losses of isoflavones occur in okara separation, about 12%, and during pressing about 44% while the remaining 33% was associated with the soluble proteins in the tofu (Wang and Murphy, 1996), but these results are determined by the amount initially present in the soy grain.

Total amounts of the three forms of isoflavones normalized for their molecular weights resulted in the same amounts in treated and control tofus (Table 3). Total daidzein was 29.93 mMol/100 g; total genistein 45.28 mMol/100 g and total glycitein 16.96 mMol/100 g of tofu. The total amount of isoflavones was then 92.16 mMol/100 g of tofu.

Table 3 – Isoflavones composition (mMol/100 g, db) of tofus.

Isoflavone	Control tofu	Treated tofu
Total Daidzein	29.89 ±0.43 [a]	29.97 ± 1.12 [a]
Total Glycitein	16.22 ± 0.48 [a]	17.70 ± 0.81 [a]
Total Genistein	45.99 ± 0.96 [a]	44.56 ± 1.06 [a]
Total Isoflavones	92.09 ± 0.93 [a]	92.23 ± 1.95 [a]

Means values in the same row followed by different letters are different (Student *t*-test, p ≤ 0.05). Total daidzein, total genistein, total glycitein, and total isoflavones were calculated by adjusting for the molecular weights of the different isoflavones using the following formulas: total daidzein= 254.23(daizin/416.36 + 6‘‘-O-malonyldaidzin/502.411 + 6‘‘-O-acetyldaidzin/458.41 + daidzein/254.23), total genistein = 270.23(genistin/432.37 + 6‘‘-Omalonylgenistin/518.411 + 6‘‘-O-acetylgenistin/474.41 + genistein/270.23), total glycitein = 284(glycitin/446 + 6‘‘-O-malonylglycitin/532 + 6‘‘-O-acetylglycitin/488 + glycitein/284), and total isoflavones = total daidzein + total genistein + total glycitein.

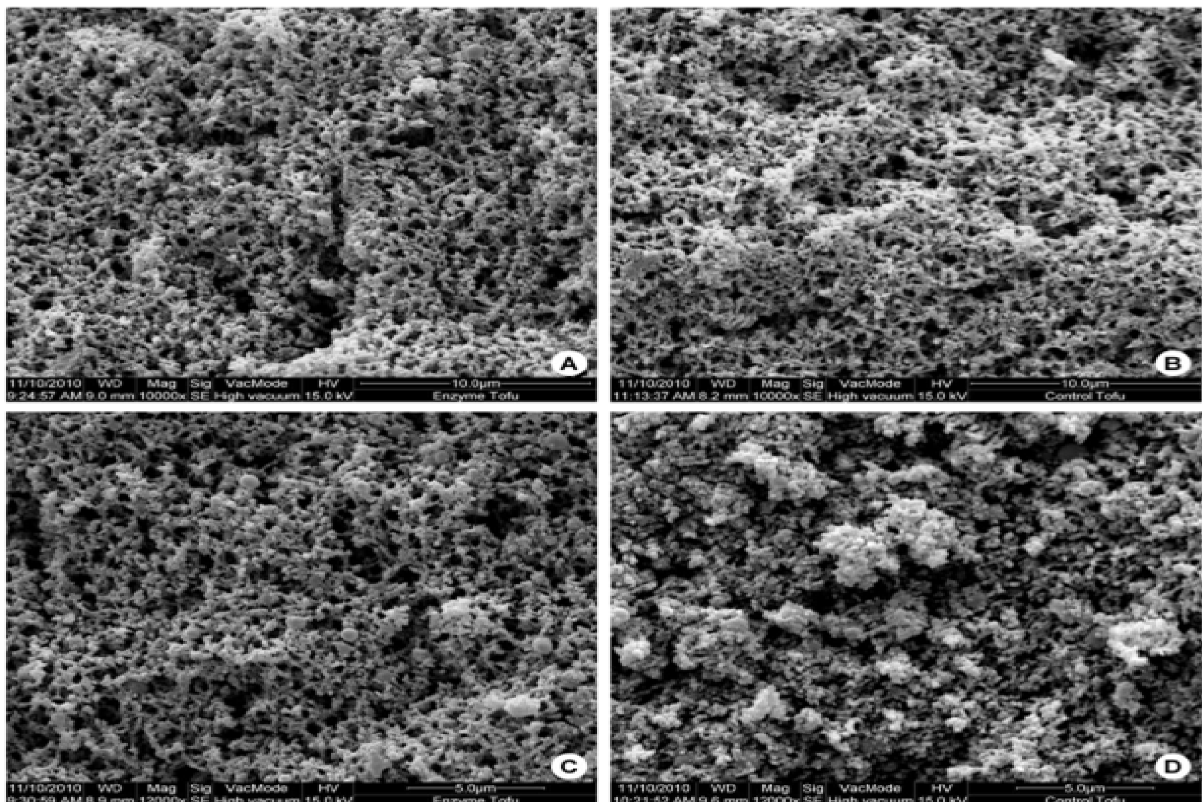
3.3 Scanning Electron Microscopic Structure

Under high magnification (10.000 and 12.000X) both tofu samples had a globular microstructure of protein aggregates and showed a fibrous three-dimensional network structure (Figure 3). Fine structures of tofu could be classified

by the network density, protein aggregation and size of coagulantes. It has been reported that the denser the network and the larger the protein aggregates in the structure the harder is the tofu (Shen et al., 1991; Han and Kim, 2002).

There were differences in size and density of the particles between the tofu samples. The treated tofu was organized with a well-defined three-dimensional honeycomb-like network with empty spaces or pores (Figure 3, A and C). The predominant forces in protein aggregation in tofu are hydrophobic interactions and the open spaces are due to the liquid phase present before dehydration during sample preparation for SEM observation. The control tofu had larger protein aggregates and was more tightly connected with filaments than the treated tofu (Figure 3, B and D). This was in agreement with the report of Han and Kim (2002), where the chitooligosaccharide tofu was organized with some vacant spaces in its network and the control tofu had larger protein aggregates and was more tightly connected between protein aggregates than the chitooligosaccharide tofu.

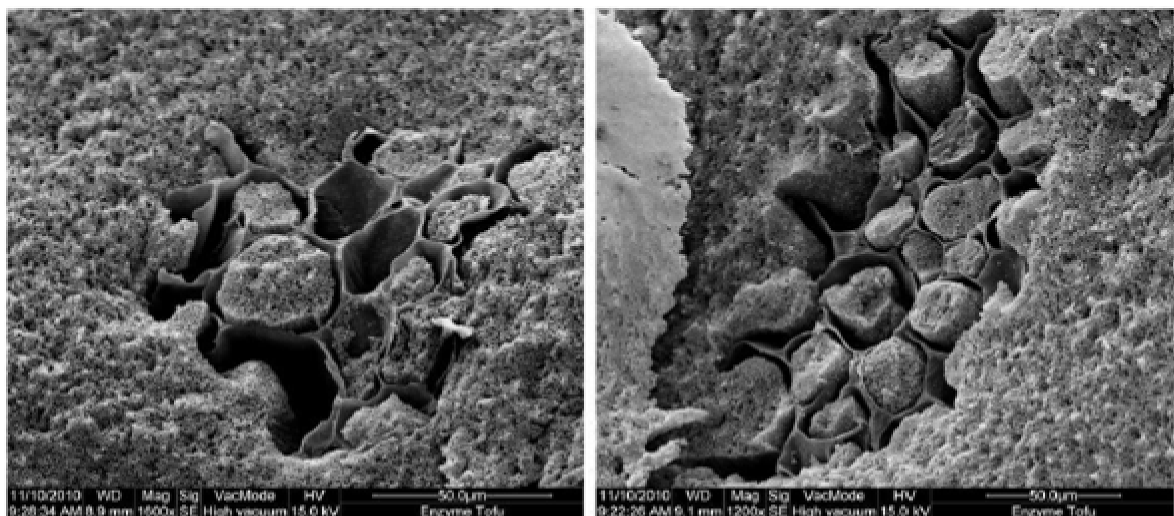
Figure 3 – Scanning electron microscopic image of tofu treated with Viscozyme L (A, C) and control tofu (B, D).



The water holding capacity (WHC) increases significantly due to decreased protein–protein interaction resulting in formation of large pores. The presence of sugars may interfere in these interactions. According to Boye, Alli, Ramaswamy, & Raghavan (1997), the WHC of gelled whey protein concentrate heated at neutral pH increased with increased sucrose concentrations. Their explanation was that sucrose decreased aggregate formation thus increased the WHC, which could be attributed to increased solvation of sucrose trapped within interstitial spaces of the whey protein gel. Gu et al. (2009) reported that the gels containing sugars were able to retain more water than the control gel; and the WHC of reducing sugar samples was higher than the sample containing non-reducing sugar.

Freeze fracturing had images which permit the exam of the protein structure and the pore distribution. A treated tofu samples in Figure 4 shows a fine protein gel as the major constituent and intact cotyledon cells as the minor constituent. The void spaces between the minor constituent represented in the micrograph by the soybean cellular walls and the major constituent (the gel) indicate that the gel contracted during sample preparation (fixation and/or critical point drying). The presence of these fragments is due to the filtration step that allowed the passage of some insoluble material through the cheesecloth.

Figure 4 – Scanning electron microscopic image of treated tofu with intact cotyledon cells.



CONCLUSION

The level of glucose, fructose and raffinose were approximately duplicated in treated tofu compared to control. The isoflavones content did not vary with enzymatic treatment and total amount of the compounds was the same for both samples. Malonyl had a higher concentration in the treated tofu while the control had more β-glucosides. Both tofu samples had a globular microstructure of protein that was integrated into clumps and showed a fibrous three-dimensional network structure.

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REFERENCES

Anon (2008). **Product sheet of Viscozyme L.** Novo Nordisk A/S, Enzymes process division, Bagsvaerd, Denmark.

Bedani, R., & Rossi, E. A. (2005). Isoflavonas: bioquímica, fisiologia e implicações para a saúde. **Boletim CEPPA**, v.23, n.2, p. 231-264.

Boye, J. I., Alli, I., Ramaswamy, H., & Raghavan, V. G. S. (1997). Interactive effects of factors affecting gelation of whey protein. **Journal of Food Science**, v.62, n.1, p. 57–65.

Carrão-Panizzi, M. C., & Bordingnon, J. R. (2000). Activity of beta-glucosidase and levels of isoflavone in soybeans cultivars affected by the environment. **Pesquisa Agropecuária Brasileira**, v.35, n.5, p. 873-878.

Coward, L., Barnes, N. C., Setchell, K. D. R., & Barnes, S. (1993). Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. **Journal of Agricultural and Food Chemistry**, v.41, n.11, p. 1961-1967.

Coward, L., Smith, M., Kirk, M., & Barnes, S. (1998). Chemical modification of isoflavones in soyfoods during cooking and processing. **The American Journal of Clinical Nutrition**, v.68, n.6, p. 1486S-1491S.

Fukutake, M., Takahashi, M., Ishida, K., Kawamurai, H., Sugimura, T., & Wakabayashi, K. (1996). Quantification of genistein and genistin in soybeans and soybean products. **Food and Chemical Toxicology**, v.34, n.5, p. 457-461.

Gu, X., Campbell, L. J., & Euston, S. R. (2009). Influence of sugars on the characteristics of glucono-δ-lactone-induced soy protein isolate gels. **Food Hydrocolloids**, v.23, v.2, p. 314–326.

Han, J. S., & Kim, M. (2002). Effects of chitooligosaccharide on the physicochemical, textural and sensory properties of tofu. **Journal of Texture Studies**, v.33, n.1, p. 1-14.

Hui, E., Henning, S. M., Park, N., Heber, D., & Go V. L. W. (2001). Genistein and daidzein/glycitein content in tofu. **Journal of Food Composition and Analysis**,v.14, n.2, p. 199-206.

Hymowitz, T., Collins, F. I., Panczner, J., & Walker, W. M. (1972). Relationship between the content of oil, protein and sugars in soybeans seed. **Agronomy Journal**, v.64, n.2, p. 613-616.

Kao, F. J., Su, N. W., & Lee, M. H. (2003). Effect of calcium sulfate concentration in soymilk on the microstructure of firm tofu and the protein constitutions in tofu whey. **Journal of Agricultural and Food Chemistry**, v.51, v.21, p. 6211-6216.

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., & Okubo, K. (1991). Malonil isoflavone in soybeans seeds (*Gyicine max* [Merrill]). **Agriculture and Biological Chemistry**, v.55, n.9, p. 2227- 2233.

Liu, K. (1997). **Soybeans:** Chemistry, Technology and Utilization, (p. 165-198). New York: Chapman & Hall.

Mandarino, J. M. G., Carrão-Panizzi, M. C., & Masuda, R. (2000). Composition content of sugars in soybean seeds of Brazilian cultivars and genotypes of Embrapa Germoplasm collection. In: **International Soybean Processing and Utilization Conference** (p. 77-78). Tsukuba: Korin Publishing.

Martínez, J. M. G. (2006). Isoflavonas en mujeres menopáusicas. **Medicina Clínica**, v.127, n.9,p. 352-356.

Masuda, R., Kaneko, K., & Yamashita, I. (1996). Sugar and cyclitol determination in vegetables by HPLC using postcolumn fluorescent derivatization. **Journal of Food Science**, v.61, n.6, p. 1186-1190.

Min, S., Yu, Y., & Martin, S. ST. (2005). Effect of soybean varieties and growing locations on the physical and chemical properties of soymilk and tofu. **Journal of Food Science**, v.70, n.1, p. C8-C12.

Mussatto, S. I., & Mancilha, I. M. (2007). Non-digestible oligosaccharides: A review. **Carbohydrate Polymers***,* v.68, n.3, p. 587–597.

Oliveira, M. A., Carrão-Panizzi, M. C., Mandarino, J. M. G., Leite, R. S., Campos Filho, P. J., & Vicentini, M. B. (2010). Quantification of the sugars, oligosaccharides and starch in special soybean genotypes/cultivars (*Glycine Max* (L) Merril) for human consumption. **Brazilian Journal of Food Technology***,* v.13, n.1, p. 23-29.

Shen, C. F., De Man, L., Buzzell, R. I., & De Man, J. M. (1991). Yield and quality of tofu as affected by soybean and soymilk characteristics: Glucono-delta-lactone coagulant. **Journal of Food Science**, v.56, n.1, p. 109-112.

STATSOFT. (2007). **Statistica for Windows**, Tulsa: Stasoft.

Taira, H. (1990). Quality of soybean for processed foods in Japan. **Japanese Agricultural Research Quarterly**, v.24, n.3, p. 224–230.

Wang, H. J, & Murphy, P. A. (1996). Mass balance study of isoflavones during soybean processing. **Journal of Agriculture and Food Chemistry***,* v.44, n.8, p. 2377-2383.

CONCLUSÕES GERAIS

O pré-tratamento enzimático de suspensões de soja resultou em tofu com diferenças na composição de mono e oligossacarídeos, de compostos fenólicos e maior atividade antioxidante em relação ao tofu controle. O tofu obtido a partir de suspensão tratada com enzima apresentou maior concentração de malonil glicosídeos enquanto a amostra controle maior quantidade de β-glicosídeos, porém o total de isoflavonas não diferiu entre as amostras. Deste modo, a atividade antioxidante dos tofus pode estar relacionada com a presença de compostos fenólicos que foram liberados pela hidrólise enzimática da parede celular.

Os tofus apresentaram diferenças sensoriais entre si, como odor de soja e uniformidade de superfície, porém não houve preferência de um em relação ao outro. Apesar do tofu tratado ter apresentado maior quantidade de glicose e frutose que o controle, não foram percebidas diferenças nos gostos das amostras. Isto pode ter ocorrido pelo fato dos tofus terem sido coagulados com glucona-delta-lactona, um coagulante ácido que pode ter mascarado o sabor doce do tofu tratado com a enzima.

O pré-tratamento para liberação de proteínas e hidrólise de carboidratos teve maior influência da temperatura e concentração de enzima, respectivamente. As micrografias dos tofus mostraram que ambas amostras apresentaram aglomerados de microestruturas globulares de proteínas e estrutura tridimensional fibrosa, típica de tofus.