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FABIANA DE OLIVEIRA MARTINS

EFFECT OF DINAMIC HIGH PRESSURE ON THE PHYSICAL, CHEMICAL AND NUTRITIONAL PROPERTIES OF OKARA

EFEITO DA ALTA PRESSÃO DINÂMICA NAS PROPRIEDADES FÍSICAS, QUÍMICAS E NUTRICIONAIS DE OKARA

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“A good head and good heart are always a formidable combination”.

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RESUMO

Okara, um subproduto de baixo valor comercial resultante do processamento do extrato hidrossolúvel de soja, é geralmente destinado à incineração ou à alimentação animal, embora seja rico em proteínas e fibras e apontado como fonte potencial de componentes bioativos. Pesquisas têm sido realizadas com foco no aproveitamento do okara, mas a sua utilização em sistemas alimentícios é limitada devido à textura desagradável que proporciona. Estudos apontam a sua desidratação como uma alternativa para a sua utilização, mas esta tecnologia resulta em grandes alterações sensoriais, nutricionais e tecnológicas, além de ser difícil devido à elevada coesividade do material. A incorporação de okara como ingrediente em alimentos é sugerida por diversos trabalho, mas eles não abordam o seu aspecto nutricional além da sua composição, como por exemplo, o efeito da tecnologia de processamento na matriz e, consequentemente, na bioacessibilidade de seus componentes. No caso do okara, não existem trabalhos que estudem o efeito da utilização de métodos não térmicos, como a homogeneização a alta pressão (HAP) nas suas propriedades reológicas e em sua microestrutura e, por conseguinte, na digestibilidade e bioacessibilidade de suas proteínas. Entre as várias vantagens, a HAP tem promovido o controle e a transformação de propriedades dos alimentos, como por exemplo, alterando texturas, convertendo fibras insolúveis em solúveis e afetando a taxa e o padrão de hidrólise proteica. O objetivo desta pesquisa foi estudar o efeito do uso de HAP (até 175 MPa, 1 a 3 passagens) nas propriedades físicas, químicas, tecnológicas, bioativas e nutricionais de uma dispersão aquosa de okara. HAP modificou a aparência de okara, tornando-o mais branco e sem partículas perceptíveis visualmente. As observações microscópicas revelaram ruptura de células e de seus fragmentos após a HAP. Com o uso de uma passagem, houve alteração do tamanho das partículas com reduções de até 82% e 92% em D[3,2], D[4,3], respectivamente, e mudanças dos parâmetros reológicos, com redução de até 66% e 85% em σ_0 e k, respectivamente e aumento de até 53% em n, com destaque para a redução da consistência da dispersão. O uso de múltiplas passagens causou mais modificações a menores pressões. A aplicação de HAP propiciou a extração de componentes de okara. A solubilidade proteica, os valores de substâncias redutoras do reagente de *Folin-Ciocalteu* e de poder de redução do íon ferro aumentaram em até 89%, 66% e 28%, respectivamente. Houve incremento de 12% na digestibilidade proteica e alteração na microestrutura e no perfil de massa molecular dos peptídeos dos digeridos. Portanto, em adição à melhoria das propriedades físicas e tecnológicas de okara, HAP mostrou ser efetivo na melhoria do potencial bioativo e das propriedades nutricionais da dispersão de okara, que possivelmente será melhor utilizada em sistemas alimentícios e será mais facilmente manipulada e transformada.

ABSTRACT

Okara, a low commercial value byproduct resulting from the processing of the water-soluble soybean extract, is generally destined to incineration or animal fed, though it is rich in protein and fibers and considered a potential source of bioactive components. Several investigations have focused on okara utilization, but its use in food systems is limited by its unpleasant texture. Some studies suggest okara dehydration as an alternative for its use, nonetheless, this technology results in massive sensorial, nutritional and technological alterations, besides being difficult due to the high cohesiveness of the material. Incorporation of okara as an ingredient in foods is suggested by numerous studies, however, none of them consider the nutritional aspect beyond its composition, for instance, the effect of the processing technology on the matrix and, consequently, on the components bioaccessibility. In the particular case of okara, there are no investigations addressing the effect of the use of non-thermal techniques, such as high-pressure homogenization (HPH), on the rheological properties and microstructure of it, and thereafter, on its protein digestibility and bioaccessibility. Amongst several advantages, HPH has presented the control and transformation of food properties through altering textures, converting insoluble in soluble fibers and affecting the rate and pattern of protein hydrolysis. The goal of this work was to study the effect of the use of HPH (until 175 MPa, 1 to 3 passages) on the physical, chemical, technological, bioactive and nutritional properties of an aqueous dispersion of okara. HPH modified the appearance of okara, making it whiter and without visually discernible particles. The microscopic observations revealed rupture of cells and their fragments after HPH. Under one passage, there was a reduction of particles size, with decrease of up to 82% and 92% in D[3,2], D[4,3], respectively, and changes in the rheological parameters with reduction up to 66% and 85% in σ_0 and k, respectively and increase up to 53% in n, highlighting the reduction of consistency dispersion. The use of multiple passages caused more modifications in lower pressures. Application of HPH caused extraction of okara components. The protein solubility, the values of Folin-Ciocalteu reducing reactive substances and the values of ferric reducing antioxidant power increased up to 89%, 66%, and 28%, respectively. There was an improvement of 12% in the protein digestibility and alteration of microstructure and molecular mass profile of peptides. Therefore, in addition to improving the physical and technological properties of okara, HPH has proven to be effective in improving the bioactive potential and nutritional property of okara dispersion, which will allow its better use in food systems and will facilitate its industrial manipulation and transformation.

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LISTA DE ABREVIATURAS E SIGLAS

0 MPa	aqueous dispersion of okara standardized to 5% protein, pre-processed and homogeneized at 0 MPa
125 MPa	aqueous dispersion of okara standardized to 5% protein, pre-processed and homogeneized at 125 MPa
175 MPa	aqueous dispersion of okara standardized to 5% protein, pre-processed and homogeneized at 175 MPa
25 MPa	aqueous dispersion of okara standardized to 5% protein, pre-processed and homogeneized at 25 MPa
75 MPa	aqueous dispersion of okara standardized to 5% protein, pre-processed and homogeneized at 75 MPa
AAPH	2,2-azobis-2-methylpropionamidine dihydrochloride
ANOVA	analysis of variance
AP	alta pressão
API	alta pressão isostática
AUC	area under the curve
b.s.	base seca
CRA	capacidade de retenção de água
D [3,2]	particle area-based diameter [µm]
D [4,3]	particle volume-based diameter [µm]
DAD	Diode Array Detector
EAG	equivalentes de ácido gálico
EHS	extrato hidrossolúvel de soja
FAEPEX	Fund for the Support of Education, Research and Extension at Unicamp
FCRRS	Folin Ciocalteau reagent reducing substances
FGF	fast green fast
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
GD	gastric digestion
HAP	homogeneização a alta pressão
HB	Herschel Bulkley
HC	homogeneização convencional

HP	homogenization pressure
HPH	high-pressure homogenization
HTA	hydrogen atom transfer
IAC	Instituto Agronômico de Campinas
ICC	intact cotyledon cell
ID	intestinal digestion
IF Southeast MG	Federal Institute of Education, Science, and Technology of the Southeast of Minas Gerais
INFABIC	National Institute of Science and Technology in Photonics Applied to Cell Biology
k	consistency index
LSM	laser scanning module
MM	molecular mass
MP	multiple passages
n	flow behaviour index
NA	numerical aperture
ND	not determined
NE	not evaluated
NR	Nile red
OCT	aqueous dispersion of okara standardized to 5% protein and pre-processed
ORAC	oxygen radical absorbance capacity
OST	aqueous dispersion of okara standardized to 5% protein without pre-processing
p	passage
PDP	perfil de distribuição de tamanho de partícula
pH	hydrogen potential
PSD	particle-size distribution
RC	reagente control
RP-HPLC	reverse phase- performance liquid chromatography
SC	sample control
SDS	sodium dodecyl sulfate
SEC-HPLC	size exclusion high-performance liquid chromatography
SET	sigle eléctron transfer mechanism

SRRFC	substâncias redutoras do reagente Folin-Cicalteu
TCA	trichloroacetic acid
TE	trolox equivalent
TFA	trifluoroacetic acid
TPTZ	2,4,6-tri 2-pyridyl) -1,3,5-triazine
trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UI	urease activity index
Unicamp	University of Campinas
WI	whiteness indice
WSSE	water-soluble soybean extract
ΔE^*	total color difference
σ	shear stress [Pa]
$\sigma 0$	yield stress [Pa]
$\dot{\gamma}$	shear rate [s^{-1}]

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CAPÍTULO 1. INTRODUÇÃO GERAL

1.1 Introdução

A soja (*Glycine max*) é uma leguminosa com excelente valor nutricional e bioativo (MEDIC; ATKINSON; HURBURGH JR, 2014). Aproximadamente 10% da soja produzida no mundo são utilizados diretamente na alimentação humana (MULTITERNO *et al*., 2017) na forma de diversos produtos, dentre eles, o extrato hidrossolúvel de soja (EHS).

Para a produção do EHS, os grãos de soja são lavados, macerados e aquecidos, sendo obtido além do EHS, um subproduto denominado okara (LI *et al*., 2012). A quantidade e a composição química do okara dependem da matéria prima, do método de extração e da proporção de soja: água utilizada (LI; QIAO; LU, 2012), mas estima-se que com 1 kg de grãos obtém-se de 6 a 9 L de EHS e aproximadamente de 1,1 a 1,8 kg de okara fresco (O'TOOLE, 2004). Anualmente, no mundo, são geradas cerca de quatro milhões de toneladas de okara, cuja maior parte é usada na alimentação animal ou descartada (VONG; LIU, 2016).

Okara apresenta em base seca (b.s.), em média, 42,5% de fibras, 37% de proteínas, 13% de lipídeos, 4,7% de carboidratos solúveis e 2,8% de cinzas (BOWLES; DEMIATE, 2006). As proteínas de okara destacam-se pela boa qualidade nutricional e tecnológica (CHAN; MA, 1999; LI *et al*., 2013; PUECHKAMUT; PANYANTHITIPONG, 2012). Também estão presentes no okara vitaminas e compostos fitoquímicos como compostos fenólicos, coumestanos e fatores anti-nutricionais (BOWLES; DEMIATE, 2006; JIMÉNEZ-ESCRIG *et al*., 2008; MA *et al*., 1997; QUITAIN *et al*., 2006; OHNO; ANO; SHODA, 1996; STANOJEVIC *et al*., 2013;). Pesquisas revelaram que componentes do okara podem desempenhar funções bioativas, tais como minimizar os sintomas da menopausa e reduzir os riscos de doenças cardiovasculares, câncer, osteoporose, hiperglicemia, hiperlipidemia e atuar como antioxidantes (HASLER, 1998; HEAD, 1997; JACKSON *et al*., 2002; JIMÉNEZ-ESCRIG *et al*., 2008; SANTOS; BEDANI; ROSSI, 2004; VILLANUEVA *et al*., 2011; ZIEGLER, 2004).

Como okara possui relevante qualidade nutricional e potencial bioativo (CERVANTES *et al*., 2010; LI; QIAO; LU, 2012; O'TOOLE, 1999; PINTO; CASTRO, 2008) e que grande parte deste resíduo não é aproveitado pela indústria de alimentos, várias pesquisas têm sido realizadas para estimular a sua utilização na alimentação humana (LI, QIAO, LU, 2012). O aproveitamento do okara provocará redução da poluição ambiental e/ou dos custos de tratamento, além da diversificação da produção ao incorporá-lo como ingrediente com elevado

valor nutricional e potencial bioativo (LI, QIAO, LU, 2012; MATEOS-APARICIO *et al*., 2010). A maioria dos estudos apontam o seu aproveitamento na forma desidratada para incorporação como ingrediente em formulações com objetivo de melhorar o valor nutricional dos produtos. A desidratação de okara por método convencional é apontada como uma tecnologia que resulta em grandes alterações sensoriais, nutricionais e tecnológicas (MULTITERNO *et al*., 2017), sendo difícil de ser conduzida devido à elevada coesividade do material (TARUNA; JINDAL, 2002) e que apresenta elevado custo (VONG; LIU, 2016). A utilização de okara como ingrediente é dificultada pelo seu alto conteúdo de fibra e a sua textura desagradável (KASAI *et al*., 2004). Além disso, as pesquisas que visam o seu aproveitamento como ingrediente não têm investigado o seu valor nutricional considerando conceitos de bioacessibilidade e sua relação com a matriz do alimento, especialmente em função da tecnologia de processamento utilizada.

O setor alimentício tem considerado a importância da matriz do produto na determinação do valor nutricional de um alimento ao desenvolver suas formulações e estabelecer as condições de processamento (PARADA; AGUILERA, 2007; TURGEON; RIOUX, 2011). A organização da matriz e o processamento podem influenciar a digestão de nutrientes (TURGEON; RIOUX, 2011; VALLE; SOUCHON; ANTON, 2013) de forma positiva ou negativa (MAT *et al*., 2016). A estrutura de alimentos à base de plantas pode ter um efeito importante na digestão (BHATTARAI *et al*., 2018), pois a parede celular limita a bioacessibilidade de nutrientes (SINGH; GALLIER, 2014). No caso de proteínas, aquelas encapsuladas dentro de paredes celulares não são hidrolisados pelas enzimas digestivas humanas (BHATTARAI *et al*., 2018). Além disso, fatores como viscosidade e conteúdo de fibra podem modular a cinética de digestão das proteínas e a liberação de peptídeos e aminoácidos no plasma (FAFAUNGWITHAYAKUL; HONGSPRABAHAS; HONGSPRABAHAS, 2011; TURGEON; RIOUX, 2011). A liberação de proteínas durante a digestão depende da sua estrutura nativa, das interações que elas estabelecem na matriz do alimento naturalmente ou em decorrência do processamento ou que acontecem no trato gastrointestinal (SINGH; GALLIER, 2014).

A alta pressão (AP) pode ser empregada em sistemas isostático (alta pressão isostática) ou dinâmico (homogeneização a alta pressão - HAP), sendo este último uma tecnologia promissora na indústria de alimentos por se basear em princípios da homogeneização convencional, só que em níveis de pressão mais elevados. A tecnologia de alta pressão tem produzido importantes avanços no conhecimento de tecnologias de conservação e

transformação dos alimentos por promover a obtenção de produtos seguros, que mantém ou melhoram as suas características sensoriais e nutricionais (MOR-MUR, 2010). AP pode ser utilizada para inativar enzimas e micro-organismos e também para alterar as propriedades sensoriais e funcionais de vários componentes dos alimentos (YORDANOV; ANGELOVA, 2010). Pode proporcionar inativação de fatores antinutricionais (GUERRERO-BELTRÁN *et al*., 2009), redução de off-flavor e melhoria de propriedades tecnológicas em leguminosas (ESTRADA-GIRÓN; SWANSON; BARBOSA-CÁNOVAS, 2005).

As propriedades sensoriais, funcionais (YORDANOV; ANGELOVA, 2010) e a digestibilidade de proteínas podem ser modificadas pela AP (ESTRADA-GIRÓN; SWANSON; BARBOSA-CÁNOVAS, 2005). Novas texturas em alimentos podem ser obtidas por meio do controle ou modificação de interações entre proteínas e polissacarídeos usando AP (TORREZAN; CRISTIANINI, 2005), pois as interações entre estes componentes exercem influência direta sobre as propriedades macroscópicas dos alimentos, como fluidez, estabilidade e textura (TORREZAN *et al*., 2007). AP também pode ser utilizada para extrair e modificar a funcionalidade de fibras dietéticas dos alimentos devido às alterações que o processo causa em sua estrutura (TEJADA-ORTIGOZA *et al*., 2015). AP pode causar a modificação da fibra insolúvel a solúvel (HUANG et al., 2015) o que pode melhorar as suas propriedades físico-químicas como capacidades de retenção de água, de óleo e de inchamento (HUANG *et al*., 2015; MATEOS-APARICIO; MATEOS-PEINADO; RUPÉREZ, 2010; ULBRICH; FLÖTER, 2014).

Portanto, o objetivo deste projeto foi estudar o efeito do uso da HAP nas propriedades físicas, químicas, tecnológicas, bioativas e nutricionais de okara. Foram pesquisadas possíveis alterações na microestrutura e no comportamento reológico de okara e na digestão gastrointestinal *in vitro* de suas proteínas após a aplicação da HAP.

1.2 Hipótese

A homogeneização sob alta pressão modifica a microestrutura, as propriedades reológicas de okara e a hidrólise de suas proteínas. Esta tecnologia transformará okara em um material com propriedades reológicas, bioativa e nutricional melhoradas, rico em fibras e em proteínas de excelente qualidade, que poderá ser utilizado como ingrediente em sistemas alimentícios ou poderá ser melhor manipulado em operações necessárias ao seu processamento na indústria de alimentos.

CAPÍTULO 2. REVISÃO BIBLIOGRÁFICA

2.1 Soja

A soja (*Glycine max*) é uma leguminosa utilizada na alimentação humana há milênios (MEDIC *et al*., 2014). A sua produção no mundo é crescente e o Brasil se destaca como o segundo maior produtor, com 116,996 milhões de toneladas das 336,699 milhões de toneladas produzidas na safra mundial de 2017/2018 (EMBRAPA, 2018). Aproximadamente 10% da soja produzida no mundo é utilizada diretamente na alimentação humana (MULTITERNO *et al*., 2017), na forma de diversos produtos como óleo, farinha, farelo, concentrado e isolado proteicos, proteína texturizada, shoyu, soja hortaliça, brotos, pasta fermentada, tofu e extrato hidrossolúvel de soja (EHS) (EMBRAPA, 2014; SILVA *et al*., 2006).

2.1.1 Microestrutura, composição, valor nutricional e bioatividade da soja

O grão de soja é composto de três principais partes: os eixos embrionários, a casca, e os cotilédones, que constituem, respectivamente, 2, 8 e 90% do grão (RIAHI; RAMASWAMY, 2003). A casca e os cotilédones contêm aproximadamente 7 e 43% de proteína, 0,3 e 23% de lipídeos, 87 e 29% de carboidratos e, 5 e 5% de cinzas, respectivamente (BAIR,1979; FERBERG; CABRAL, 2001). A Fig. 2.1 mostra um esquema que resume as estruturas que compõem a casca e os cotilédones.

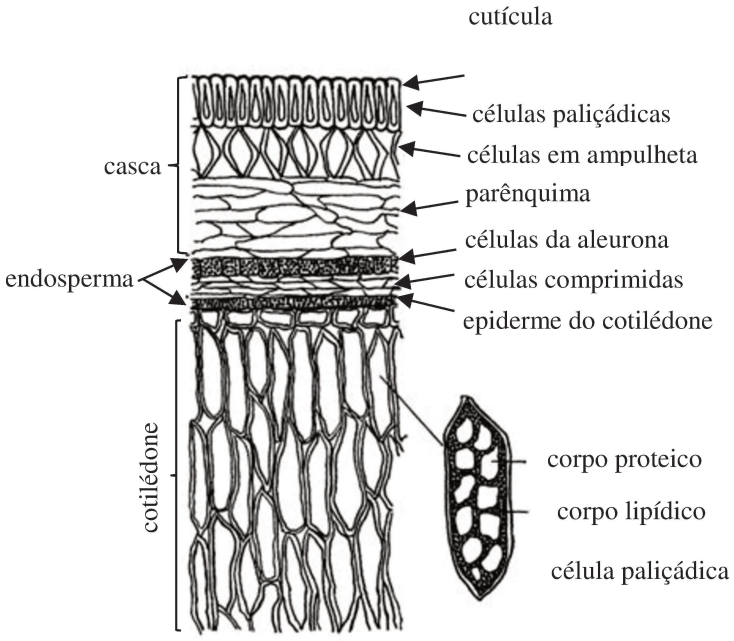

Fig. 2.1 Seção transversal da casca e de parte do cotilédone de soja madura.
Fonte: Adaptado de Bair (1979).

Os cotilédones são compostos por várias células paraquimentosas do tipo paliçada, possuem 15-20 mm de diâmetro e 70-80 mm de comprimento e são preenchidas predominantemente por proteína e lipídeo, sendo este imerso em uma rede citoplasmática (Fig. 2.2). A proteína é empacotada em inclusões subcelulares de forma esférica, que são denominadas corpos de proteína ou grãos de aleurona e possuem 2-10 µm de diâmetro. Os corpos proteicos contém 70-80% da proteína total da soja, possuem tamanho médio de 8-10 mm, com variações na faixa de 2-20 mm (ROSENTHAL; PYLE; NIRANJAN, 1998), forma irregular e mostram uma associação próxima de corpos lipídicos à sua superfície (BAIR, 1979). Os lipídeos, denominados corpos lipídicos, lipossomos ou esferossomos, possuem de 0,2 a 0,5 µm (RIAHI; RAMASWAMY, 2003; ROSENTHAL; PYLE; NIRANJAN, 1998), são redondos ou ovais, distribuídos por todo o citoplasma, tipicamente ao redor de corpos de proteína e nas margens do citoplasma (BAIR, 1979), possuem de 0,2 a 0,5 mm de diâmetro e são envoltos por uma membrana fosfolipídica intercalada com proteínas oleosinas (ROSENTHAL; PYLE; NIRANJAN, 1998). A região de perímetro espesso que circunda a célula é a parede celular, descrita como uma rede robusta (PREECE *et al*., 2015) composta de duas estruturas, as paredes primária e secundária. A parede primária contém pectinas, hemiceluloses e celulose reticulada com proteína e a secundária é composta de celulose e hemicelulose que também são capazes de ligar proteínas (CAMPBELL *et al*., 2011; ROSENTHAL; PYLE; NIRANJAN, 1998). As células são mantidas juntas por substâncias adesivas compostas por pectinas e proteínas ricas em glicina e hidroxiprolina que ficam localizadas no espaço extracelular entre elas (CAMPBELL *et al*., 2011).

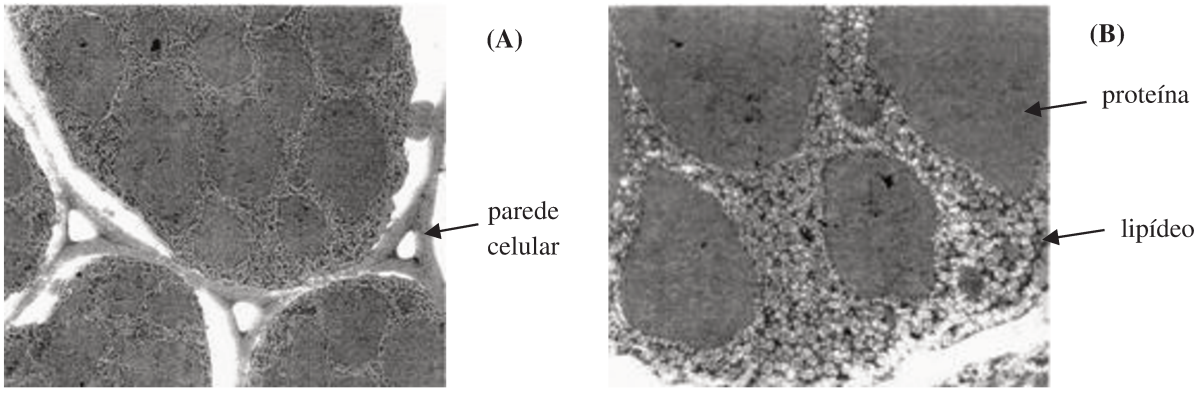

Fig. 2.2. Fotomicrografias eletrônicas de transmissão do tecido cotiledonar de soja (A) ampliação x 3200; (B) ampliação x 8200.

Fonte: Adaptado de Rosenthal; Pyle; Niranjan (1998).

A soja é componente importante na nutrição humana e animal por apresentar, em quantidade e qualidade, principalmente proteínas e lipídeos, além fibras e de outros compostos com valor bioativo (O'KEEFE; BIANCHI; SHARMAN, 2015). A composição química da semente de soja varia devido a fatores genéticos e influências do meio ambiente, mas uma composição média pode ser esperada (SANTOS *et al*., 2007). A soja brasileira, integral e seca, possui em 100 g de porção comestível, 451 kcal, 8,3 g de água, 33,6 g de proteína, 30,7 g de carboidratos, sendo 17,3 g de fibras dietéticas totais (1,0 g de fibra solúvel e 16,3 g de fibra insolúvel), 22,6 g de lipídeos (sendo 3,14 g de ácidos graxos saturados, 5,02 g de ácidos graxos monoinsaturados e 11,13 g de ácidos graxos poli-insaturados), 4,8 g de cinzas, 1800 mg de potássio, 580 mg de fósforo, 250 mg de cálcio, 250 mg de magnésio, 9,0 mg de ferro, 3,5 mg de zinco, 2,54 mg de manganês, 2 mg de sódio, 1,11 mg de cobre, 7,2 mg de vitamina E, 2,2 mg de niacina, 1,68 mg de ácido pantotênico, 0,77 mg de vitamina B1, 0,45 mg de vitamina B6, 0,29 mg de vitamina B2, 220 µg de folato, 36 µg de vitamina K, 15 µg de caroteno, 3 µg de retinol equivalente e traços de ácido ascórbico (MEXT, 2015).

Além de seu valor nutricional, a soja tem sido reconhecida pelos efeitos benéficos que pode proporcionar à saúde, como a redução de riscos de doenças crônicas e degenerativas. Estes benefícios são relacionados aos seus diversos compostos, como proteínas, ácidos graxos poli-insaturados (ULIANA; VENTURINI FILHO; ULIANA, 2012) e também a compostos fitoquímicos, como saponinas, fitoesterois, isoflavonas (genisteína e daidzeína), lignanas, lecitinas, oligossacarídeos, inibidores de tripsina e fitatos, alguns com efeitos benéficos à saúde humana e animal (BOWLES; DEMIATE, 2006; JIMÉNEZ-ESCRIG *et al*., 2008; QUITAIN *et al*., 2006; STANOJEVIC *et al*., 2013).

Inúmeras pesquisas comprovam ou sugerem a relação entre os componentes da soja e a prevenção de sintomas da pré e pós menopausa nas mulheres, doenças cardiovasculares, Alzheimer, risco dos cânceres de próstata, cólon e mama e doenças ósseas como a osteoporose, além da potencial atividade antioxidante, anti-obesidade, e dos efeitos hipocolesterolêmico, antiviral e imunoregulatoria (BARBOSA *et al*., 2006; HASLER, 1998; HEAD, 1997; JACKSON *et al*., 2002; LI; QIAO; LU, 2012; ZIEGLER, 2004).

2.1.1.1 As proteínas da soja

A soja contém uma mistura de proteínas nas quais se incluem as de armazenamento (β-conglicinina, glicinina, albumina), enzimas (lipoxigenase, chalcona sintase, catalase, urease), inibidores enzimáticos (inibidores de Bowman-Birk e Kunitz), proteínas glicosiladas

(lectina) e peptídeos (lunasina) (WANG *et al*., 2008). As proteínas da soja são classificadas em função do coeficiente de sedimentação quando centrifugadas a pH 7,6 e forca iônica 0,5 em frações: 2S (inibidores de tripsina, Kunitz e Bowman Birk, e citocromo C), 7S (β-conglicilina), 11S (glicinina) e 15S (hemaglutininas e enzimas, amilase, lipoxigenase, urease), sendo que as frações 7S e 11S são maioria e representam 90% das proteínas (IWABUCHI; YAMAUCHI, 1987).

A fração 7S (β-conglicilina) possui MM de 150 a 200 kDa e é composta por três subunidades de polipeptídios diferentes α' (57-83 kDa), α (57-76 kDa) e β (42-53 kDa), que interagem para formar 6 isômeros conhecidos, designados como B1 a B6. As subunidades α, α' e β são ricas em asparginina, glutamina, leucina e arginina, as subunidades α e α' são ricas em cisteína e pobres em metionina, a subunidade β (glicoproteína) contém 4-5% de carboidratos e não contém metionina. A fração 11S (glicinina) possui MM de 320 a 375 kDa e é uma proteina simples composta de seis subunidades de polipeptídios ácidos e básicos ligados por ligação dissulfeto (37 a 45 kDa e 18 a 20 kDa, respectivamente) (NAKORNPANOM; HONGSPRABHAS; HONGSPRABHAS, 2010; WANG *et al*., 2008).

As proteínas da soja contribuem de maneira significativa com o valor nutricional desta leguminosa. Elas possuem composição similar às proteínas de origem animal (BANASZKIEWICZ, 2011) e dentre as leguminosas, apresenta a maior pontuação e um conjunto de aminoácidos mais próximo do padrão da FAO (Food and Agriculture Organization) e da WHO (World Health Organization), com teor elevado de lisina, fornecendo todos os aminoácidos essenciais para humanos, embora metionina e cisteína sejam aminoácidos limitantes (BURSSENS *et al*., 2011; O'KEEFE; BIANCHI; SHARMAN, 2015; SALGADO; DONADO-PESTANA, 2011).

As proteínas da soja destacam-se também devido às excelentes características para inclusão em alimentos, como capacidade gelificante, emulsionante e de retenção de água e óleo (NISHINARI *et al*., 2014). Além dos benefícios nutricionais e tecnológicos, as proteínas, peptídeos e aminoácidos da soja podem apresentar bioatividade, como atividade anti-hipertensiva (O'TOOLE, 1999), hipocolesterolêmica (SANTOS *et al*., 2004; XU *et al*., 2000) e antioxidante (AMIN; MUKHRIZAH, 2006; YOKOMIZO; TAKENAKA; TAKENAKA, 2002). Com base em evidências sobre os benefícios à saúde demonstrados, as proteínas da soja ganharam reconhecimento por diversas agências de saúde (JAEKEL; RODRIGUES; SILVA, 2010). No Japão, além de isoflavonas, oligossacarídeos e fitoesterol, as proteínas são usadas como componentes bioativos e nos Estados Unidos da América e no Brasil recomenda-se a

ingestão de 25 g de proteína de soja por dia para contribuir com a redução do colesterol (BRASIL, 2016; SANTOS; BEDANI; ROSSI, 2004).

2.2 Okara

Okara é um resíduo abundante e barato gerado na produção de EHS (LI; QIAO; LU, 2012; MULTITERNO *et al*., 2017; PÉREZ-LÓPEZ; MATEOS-APARICIO; RUPÉREZ, 2017; SUREL; COUPLET, 2005). O processamento do EHS pode ser realizado a partir de diversas tecnologias que derivam do método tradicional chinês (O'TOOLE, 2004), cujo fluxograma está apresentado na Figura 2.3. Uma etapa opcional, porém muito comum no processamento no processo, é o descascamento, que visa proporcionar soja com maior teor proteico e menor teor de fibras, além diminuir o tempo de maceração dos grãos (FELBERG; CABRAL, 2001).

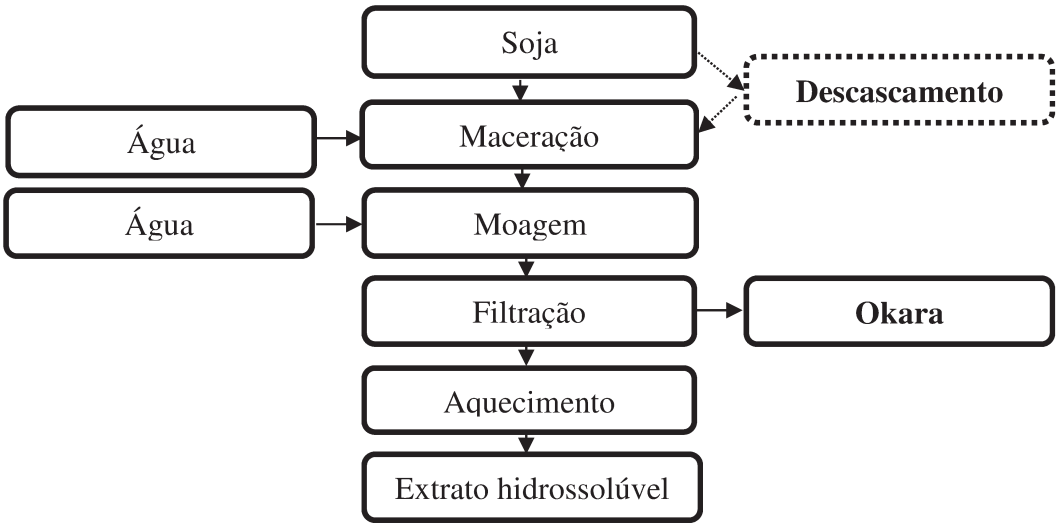

Fig. 2.3. Fluxograma geral de processamento de extrato hidrossolúvel de soja.
Fonte: adaptado de O'Toole (2004).

A quantidade de resíduo gerado depende da matéria prima e do método de processamento, mas em média, com 1 kg de grãos obtém-se de 6 a 9 L de EHS e de 1,1 a 1,8 kg de okara (O'TOOLE, 2004). Estima-se que são gerados a cada ano, globalmente, cerca de quatro milhões de toneladas de okara (VONG; LIU, 2016).

Embora seja um resíduo com relevante qualidade nutricional e potencial bioativo (CERVANTES *et al*., 2010; LI *et al*., 2012; O'TOOLE, 1999; PINTO; CASTRO, 2008), não é

devidamente aproveitado, pois grande parte é usada na alimentação animal ou descartada, sendo pouco comum o seu uso industrial (SUREL; COUPLET, 2005). O aproveitamento do okara pode ser uma alternativa para as indústrias de EHS reduzirem a poluição ambiental e os custos com tratamento, diversificarem a produção (CIABOTTI *et al*., 2009; LI; QIAO; LU, 2012; MATEOS-APARICIO *et al*., 2010) e diminuirem o desperdício de compostos bioativos de grande interesse (JANKOWIAK *et al*., 2014). Por isso, várias pesquisas têm sido realizadas para estimular o seu aproveitamento na alimentação humana (LI; QIAO; LU, 2012). Os estudos mostram principalmente sua utilização na forma *in natura*, como ingrediente em, por exemplo, pães, biscoitos, cereais, bebidas, macarrão, produtos carnes e molhos; na forma processada, como um produto desidratado ou fermentado; ou ainda como matéria prima para obtenção de componentes úteis como álcoois, ácidos, enzimas, vitaminas, por exemplo (BOWLES; DEMIATE, 2006; CERVANTES; AOKI; ALMEIDA, 2010; LI; QIAO; LU, 2012; PINTO; CASTRO, 2008).

No entanto, como okara é um material particulado heterogêneo, a sua incorporação na forma *in natura* em alimentos sem o comprometimento de atributos sensoriais, principalmente a textura, é difícil (WEI *et al*., 2018). A secagem, embora seja uma alternativa para transformação de okara em um ingrediente com vida de prateleira estendida e de fácil transporte (VONG *et al*., 2017), pode resultar em produto com qualidade muito reduzida quando comparado ao produto original (MULTITERNO *et al*., 2017), além de estudos apontarem que o alto custo do processo ultrapassa o valor da proteína (VONG; LIU, 2016) e de haver uma dificuldade de secagem devido a elevada coesividade do material que é muito úmido (TARUNA; JINDAL, 2002). Já a fermentação, além de modificar as características sensoriais de okara, pode causar perdas nutricionais como diminuição de aminoácidos essenciais e também afetar as propriedades funcionais de okara (VONG; LIU, 2016).

2.2.1 Composição de okara e seu potencial bioativo

O processo de produção do EHS não é capaz de extrair todos os nutrientes da soja, o que resulta em um resíduo rico em fibras e compostos fitoquímicos, além das proteínas, presentes em elevado teor, mas com reduzido valor comercial (CERVANTES *et al*., 2010; O'TOOLE, 1999; PINTO; CASTRO, 2008).

A composição química do okara depende do cultivar de soja, do método e da eficiência de extração, mas uma composição média é estimada (VONG; LIU, 2016; O'TOOLE, 2004; O'TOOLE, 1999). Bowles & Demiate (2006) pesquisaram a composição do okara e

constataram em base seca (b.s.): 42,5 g/100 g de fibras alimentares e 4,7 g/100 g de carboidratos solúveis, 37,0 g/100 g de proteínas, 13,0 g/100 g de lipídeos e 2,8 g/100 g de cinzas. Os carboidratos solúveis eram transferidos em grande parte para o EHS, as fibras alimentares ficavam quase totalmente retidas no okara, enquanto os teores dos demais componentes eram similares.

A quantidade de carboidratos (b.s.) de okara varia entre 32,6 g/100 g (RASHAD *et al*., 2011) e 59,6 g/100 g, sendo que 92% são classificados como fibras (MATEOS-APARICIO et al., 2010). As fibras são os polissacarídeos não-amiláceos compostos por fração de fibras insolúvel (85%) e solúvel (7%) (Mateos-Aparicio *et al*., 2010), que compreendem celulose, polímeros não-celulósicos e polissacarídeos pécticos (ramngalacturonanas tipos I e II, arabinogalactanas I e xilogalacturonanas) (CHOCT; DERSJANT-LI; PEISKER, 2010). A quantidade de fibra em okara pode variar de 14,5 a 55,4 g/100g. Okara possui aproximadamente 7% de carboidratos de baixa MM como inulina, estaquiose, rafinose, sacarose, glucose, galactose, arabinose e frutose okara. O amido está presente em quantidade inferior a 1% (MATEOS-APARICIO *et al*., 2010). O okara apresenta o dobro (55,48 g/100 g) de fibras alimentares comparado aos grãos de soja (24,36 g/100 g). A fibra insolúvel é a fração majoritária tanto em okara (50,77 g/100 g) como nos grãos de soja (20,86 g/100 g). A fibra solúvel está em menor quantidade em okara (4,71 g/100 g) e em grãos de soja (3,50 g/100 g) (MATEOS-APARICIO *et al*., 2010).

No processamento do EHS, em média, 80% das proteínas da soja ficam no extrato (O'TOOLE, 2004; WALISZEWSKI *et al*., 2002) e, portanto, 20% são retidas em okara. Compõe este grupo uma mistura de proteínas de armazenamento da semente (β-conglicinina, glicinina, albumina), enzimas (lipoxigenase, chalcona sintase, catalase, urease), inibidores enzimáticos (inibidores de Bowman-Birk e Kunitz), proteínas glicosiladas (lectina) e peptídeos (WANG *et al*., 2008). O teor de proteina (b.s.) varia entre 24 a 40% (BOWLES; DEMIATE, 2006; JIMÉNEZ-ESCRIG *et al*., 2008; RASHAD *et al*., 2011). A fração proteica de okara apresenta aminoácidos indispensáveis como histidina, isoleucina, leucina, lisina, metionina, fenilalanina, treonina e valina, além de também apresentar ácido aspártico, serina, ácido glutâmico, glicina, alanina, cisteína, tirosina, arginina e prolina (CHAN; MA, 1999). O seu bom perfil de aminoácidos é reconhecido como o mais completo de todas as fontes vegetais, com um padrão de aminoácidos que se assemelha ao das proteínas animais de alta qualidade, exceto para cisteína, metionina e tirosina (MA, 2004). As proteínas de okara apresentam taxa de eficiência proteica (2,71) superior a de outros produtos de soja, como por exemplo, o EHS

(2,11) (DAJANTA *et al*., 2011). As proteínas se destacam por sua qualidade nutricional e tecnológica, que são comparáveis a isolados proteicos de soja comerciais (CHAN; MA, 1999; LI *et al*., 2013; MA *et al*., 1997; PUECHKAMUT; PANYATHITIPONG, 2012).

A fração lipídica em okara varia entre 8,5 e 20% (b.s.) (JIMÉNEZ-ESCRIG *et al*., 2008; MATEOS-APARICIO *et al*., 2010), sendo ela composta de ácidos graxos insaturados (linoleico, linolênico e oleico) e saturados (palmítico e esteárico), com destaque para o ácido linoleico, que representa 54% do conteúdo total de ácidos graxos (JIMÉNEZ-ESCRIG *et al*., 2008). O teor de cinzas é próximo a 4% (b.s.) (BOWLES; DEMIATE, 2006; MATEOS-APARICIO *et al*., 2010; RASHAD *et al*., 2011;), sendo que os minerais presentes (b.s.) são: potássio (1,35 mg/100 g), sódio (0,03 mg/100 g), cálcio (0,32 mg/100 g), magnésio (0,13 mg/100 g), ferro (0,62 mg/100 g), cobre (0,10 mg/100 g), manganês (0,21 mg/100 g), zinco (0,29 mg/100 g) (MATEOS-APARICIO *et al*., 2010). Os teores de cálcio, principalmente, além de ferro e zinco descritos em okara, são relativamente altos em comparação com outras fontes vegetais (O'TOOLE, 1999). Estão presentes em okara as seguintes vitaminas: vitamina E (0,7 mg/100 g), ácido pantotênico (0,31 mg/100 g), niacina (0,2 mg/100 g), vitamina B1 (0,11 mg/100 g), vitamina B6 (0,06 mg/100 g), vitamina B2 (0,03 mg/100 g), folato (14 µg/100 g) e vitamina K (8 µg/100 g) (MEXT, 2015). Estão presentes pigmentos da soja como carotenoides e clorofilas, que caracterizam a coloração amarelada do okara (MARKLEY; GOSS, 1944).

Okara possui fatores antinutricionais como saponinas, fitatos, fosfolipídios, inibidores de proteases, inibidores de fitases e inibidores de tripsina (HEAD, 1997; JIMÉNEZ-ESCRIG *et al*., 2008; QUITAIN *et al*., 2006;). Além da composição, a determinação de fatores antinutricionais também pode contribuir para a avaliação da qualidade nutricional dos produtos de soja, pois a presença deles pode comprometer a digestibilidade de tais produtos para humanos e animais (PINTO; CASTRO, 2008). No entanto, é possível produzir EHS e, consequentemente, okara com níveis aceitáveis de atividade de inibidores e ao mesmo tempo reter o valor nutricional destas proteínas que são ricas em cisteína (STANOJEVIC *et al*., 2013).

Coumestanos e compostos fenólicos também estão presentes em okara (BOWLES; DEMIATE, 2006; JIMÉNEZ-ESCRIG *et al*, 2008; OHNO; ANO; SHODA, 1996; QUITAIN; ORO; KATOH; MORIYOSHI, 2006; STANOJEVIC *et al*., 2013). Especificamente, as isoflavonas se destacam como importantes fenólicos da soja e no processamento do EHS, aproximadamente 31% do seu conteúdo é transferido para okara (BARBOSA *et al*., 2006; JACKSON *et al*., 2002), o que corresponde a aproximadamente 35,73 g/100 g (BOWLES; DEMIATE, 2006). Doze componentes de isoflavona têm sido isolados de soja, sendo 3

agliconas (daidzeína, genisteína e gliciteína) e os outros nove, são seus respectivos glicosídeos conjugados (JACKSON *et al*., 2002).

Diversos componentes de okara têm sido relacionados às várias funções fisiológicas e terapêuticas, tais como redução dos riscos de doenças cardiovasculares, câncer, osteoporose, sintomas da menopausa, hiperglicemia, hiperlipidemia e atividade antioxidante (AMIN; MUKHRIZAH, 2006; BARBOSA *et al*., 2006; HASLER, 1998; HEAD, 1997; LI; QIAO; LU, 2012; JIMÉNEZ-ESCRIG *et al*., 2008; JACKSON *et al*., 2002; O'TOOLE, 1999; SANTOS; BEDANI; ROSSI, 2004; VILLANUEVA *et al*., 2011; ZIEGLER, 2004;). Vários estudos indicam que o okara é fonte de compostos antioxidantes (AMIN; MUKHRIZAH, 2006; MATEOS-APARICIO *et al*., 2010; NAEEM *et al*., 2015; STANOJEVIC *et al*., 2014; JIMÉNEZ-ESCRIG *et al*., 2010; JIMÉNEZ-ESCRIG *et al*., 2008; PARADA; AGUILERA, 2007; QUITAIN *et al*., 2006; SBROGGIO *et al*., 2016; WALISZEWSKI; PARDIO; CARREON, 2002; WU *et al*., 2012).

A atividade antioxidante tem sido estudada em função do reconhecido papel que exerce na proteção contra danos oxidativos, o que representa um papel preventivo contra doenças crônicas como as cardiovasculares, câncer, diabetes, infecções e asma, por exemplo e, consequentemente uma ação promotora de saúde (PANDEY; RIZVI, 2009). Além disso, substâncias antioxidantes podem atuar nos alimentos estendendo a sua vida de prateleira por meio da prevenção da peroxidação lipídica, evitando o aparecimento de odores e sabores desagradáveis e a redução do valor nutricional (MOURE *et al*., 2001). Embora os antioxidantes naturais frequentemente apresentem atividade antioxidante menor que os sintéticos, eles são mais vantajosos por não apresentarem efeitos adversos e por isso poderem ser usados em quantidades ilimitadas (MOURE et al., 2001).

Soja e produtos derivados contêm vários compostos fenólicos com atividade antioxidante (RASHAD *et al*., 2011). O conteúdo de fenólicos de okara pode ser relacionado com a sua capacidade de inibir ou sequestrar radicais livres, atuando possivelmente na redução de hidroperóxido, inativação de radicais livres, complexação com íons metálicos ou na combinação desses mecanismos (RASHAD *et al*., 2011). No entanto, a atividade antioxidante de subprodutos pode estar relacionada não somente com a presença de compostos fenólicos, mas também com a de outros componentes fitoquímicos (AMIN; MUKHRIZAH, 2006; MOURE *et al*., 2001) e também com a contribuição de complexos proteína-fenólico (SILVA *et al*., 2017). Salgado & Donado-Pestana (2011) destacam que devido à diversidade de

componentes presentes na soja é difícil atribuir uma função biológica a um único composto porque pode existir efeito sinergístico.

Amin & Mukhrizah (2006) revelaram em seus estudos, que frações de okara (extratos aquosos e metanólicos) apresentaram atividade antioxidante. Estudos mostram que, em okara, diversos componentes como α-tocoferol, γ-tocoferol, δ-tocoferol (MATSUO, 1997), coumestanos, saponinas, fitatos (LI *et al*., 2012), proteínas e peptídeos (WIBOONSIRIKUL *et al*., 2013), aminoácidos como cisteína, metionina, tirosina, fenilalanina e triptofano (WALISZEWSKI *et al*., 2002) também contribuem com a atividade antioxidante.

Em geral, os componentes antioxidantes de okara têm a sua liberação dificultada, pois eles estão associados com as fibras (PARADA; AGUILERA, 2007). A redução do tamanho de partícula pode colaborar para a melhoria da extração de componentes que contribuem com a atividade antioxidante. Os vegetais contêm fenólicos insolúveis e solúveis. Os solúveis estão presentes nos vacúolos das células vegetais e os insolúveis são encontrados nas paredes celulares ou em associação com outros compostos, como ácidos carboxílicos e orgânicos, aminas, lipídios e até com outros fenois (PANDEY; RIZVI, 2009; VONG; LIM; LIU, 2017). Wiboonsirikul *et al*. (2013) relataram que os fenólicos insolúveis são difíceis de serem recuperados por métodos tradicionais de extração, mas que o uso do tratamento com água subcrítica poderia provocar a desintegração da parede celular e a liberação destes componentes nos extratos, além também da liberação de outros compostos de parede celular, como aminoácidos, peptídeos, proteínas e carboidratos. Bonilla *et al*. (1999) constataram que a redução no tamanho das partículas por esmagamento mecânico de bagaço de uva favoreceu a extração de fenólicos. Meyer; Jepsein; Sϕrensen (1998) extraíram antioxidantes com auxílio de enzimas em bagaço de uva e correlacionou o rendimento da extração de fenois com a quebra da parede celular pelas enzimas utilizadas, em que a melhor extração foi devido a redução do tamanho de partícula e a melhor degradação enzimática de polissacarídeos. Kasai *et al*. (2004) relataram que a degradação da parede celular secundária de okara por protease, provavelmente, aumentou o acesso da carboidrase ao ácido fenólico ligado. Tsubaki *et al*. (2009) observaram aumento na extração de polifenois e da atividade antioxidante em okara tratado por micro-ondas em meio aquoso, e constataram por microscopia eletrônica de varredura a solubilização de materiais da parede celular.

Devido à natureza complexa dos produtos vegetais e ao fato de os antioxidantes poderem atuar por vários mecanismos - ligando íons metálicos, limpando radicais e peróxidos em decomposição ou agindo até por uma combinação de mecanismos - é recomendado que a

atividade antioxidante não seja avaliada por um único método (MOURE *et al*., 2001; SBROGGIO *et al*., 2016). Em geral, compostos antioxidantes podem agir por dois mecanismos baseados na transferência de elétrons (SET) ou de átomos de hidrogênio (HTA) (ALVES *et al*., 2010). Silva *et al*. (2017), por exemplo, mostraram que amostras proteicas atuam como antioxidantes principalmente pela transferência de hidrogênio enquanto compostos fenólicos, além deste mecanismo, atuam também pela doação de elétrons. É usual, portanto, avaliar-se os potenciais de oxidação-redução pela medida do poder de redução e da capacidade de eliminação de radicais livres (JIMÉNEZ-ESCRIG *et al*., 2010). Os métodos normalmente empregados nos ensaios de atividade antioxidante são a determinação de substâncias redutoras do reagente *Folin-Cicalteu* (SRRFC) e do poder de redução do íon ferro (FRAP) que se baseiam no mecanismo SET e determinação da capacidade de absorção do radical oxigência (ORAC) que é fundamentado no mecanismo HTA (SILVA *et al*., 2017).

O método de determinação de SRRFC é baseado na reação de transferência de um elétron do agente redutor para o molibdênio Mo^{+6} contido no complexo fosfomolíbdico, que ao ser reduzido para Mo^{+5}, tem a sua cor alterada e monitorada a 765 nm (EVERETTE *et al*., 2010; MEDINA, 2011). O resultado de SRRFC é uma medida da capacidade redutora total, pois o reagente *Folin-Ciocalteau* além de reagir com compostos fenólicos, também reage com antioxidantes não fenólicos e outras substâncias redutoras, por exemplo, aminoácidos aromáticos e proteínas que o contenham, ácido ascórbico, frutose e glicose (MEDINA, 2011).

O método de FRAP é baseado na reação de transferência de um elétron do agente redutor para o ferro contido no complexo Fe^{+3}-TPTZ (2,4,6-tris (2-piridil)-s-triazina), que ao ser reduzido a Fe^{+2}, tem a sua cor alterada e monitorada a 595 nm (RUFINO *et al*., 2006).

O método de ORAC é baseado na reação de transferência de um átomo de hidrogênio do antioxidante para o radical peroxila, que é gerado a partir do 2,2’ azobis (2-amidino-propano) dihidrocloreto (AAPH). Ao receber o hidrogênio doado, o “ataque” da molécula à fluoresceína cessa ou reduz, o que leva ao menor decaimento da fluorescência (DÁVALOS; GÓMEZ-CORDOVES; BARTOLOMÉ, 2004)

2.2.2 Solubilidade das proteínas de okara

As propriedades funcionais tecnológicas são propriedades não nutricionais (SGARBIERI, 1996) que desempenham papel importante na qualidade de um alimento (CHAUD; SGARBIERI, 2006; CHOU; MORR, 1976), influenciando as aplicações em sistemas alimentícios e a aceitabilidade do consumidor (KINSELLA, 1979). Produtos

alimentícios proteicos devem possuir alto grau de solubilidade proteica para que sejam úteis e funcionais (MA, 2004; MORR *et al*., 1985).

Tecnicamente a solubilidade proteica é um parâmetro operacional determinado pela retenção de proteína no sobrenadante após a centrifugação de uma solução a um determinado tempo e força centrífuga (VODJANI, 1996). É uma propriedade hidrofílica influenciada por fatores intrínsecos como composição, tamanho, carga, conformação, concentração, hidrofobicidade, hidrofilicidade e propriedades estéricas das proteínas e também por fatores ambientais como a presença e interação com outros componentes, pH, temperatura, força iônica e métodos de extração (KINSELLA, 1979). Esta propriedade se relaciona diretamente com outras características funcionais, como as capacidades de geleificação e de formação de espuma, o poder de emulsificação e a viscosidade (MATTIL, 1971). Geralmente as proteínas são os principais componentes funcionais nos alimentos, embora os carboidratos também possam desempenhar papel importante na capacidade de ligar água e no controle da viscosidade (KINSELLA, 1979).

Especificamente no caso do okara, a literatura descreve, entre as limitações para o seu uso, a necessidade de melhorar a solubilidade de suas proteínas (CHAN; MA, 1999). Okara possui baixa solubilidade em água (PRÉSTAMO *et al*., 2007) por ser constituído principalmente por proteínas e fibras e por possuir pequena quantidade de amido (SONGSRIROTE *et al*., 2017). A reduzida solubilidade se aplica às proteínas de okara, que são predominantemente insolúveis em água (VISHWANATHAN; SINGH; SUBRAMANIAN, 2011; MA, 2004), embora pequena parte das proteínas solúveis da soja permaneça após o processamento do EHS, dependendo do procedimento de extração utilizado e de como o EHS é removido do okara (O'TOOLE, 1999). A baixa solubilidade das proteínas em água é explicada pela forte agregação entre as proteínas através de interações hidrofóbicas, pela ligação delas com outros componentes como fibras (SASAKI *et al*., 2000; O'TOOLE, 1999) e lipídeos (SUREL; COUPLET, 2005) e também pela desnaturação proteica que pode ocorrer durante o processamento do EHS quando se utiliza tratamento térmico antes da etapa de extração (CHAN; MA, 1999; ROSENTHAL; PYLE; NIRANJAN, 1998).

A maior parte das proteínas do okara está muito ligada à estrutura da parede celular (MATEOS-APARICIO *et al*., 2010; MATEOS-APARICIO; REDONDO-CUENCA; VILLANUEVA-SUÁREZ, 2010). Mateos-Aparicio *et al*. (2010) ao isolar polissacarídeos de okara constataram que não era possível remover completamente as proteínas com o tratamento aplicado, possivelmente porque a proteína está ligada à estrutura da parede celular. No okara é

descrito a presença de duas paredes celulares existentes no grão de soja, uma primária, composta principalmente de celulose, e uma secundária, altamente estruturada composta de fibras associadas a proteínas (KASAI et al., 2004), denominadas regiões “non-egg-box” (YOSHII et al., 1996). Mateos-Aparicio *et al*. (2010) sugerem que existe forte ligação entre proteínas estruturais, celulose, hemicelulose e redes de pectinas. Isto possivelmente explica a baixa solubilidade das proteínas de okara, mesmo com o uso de hidrólise enzimática ou ácida (CHAN; MA, 1999). Esta parede celular secundária é resistente à digestão por celulases, hemicelulases, proteases e até pectinases (YAMAGUCHI; OTA; HATANAKA, 1996). Surel, Couplet (2005) destacam que as proteínas de okara também interagem com os lipídeos e que mesmo tratamentos com proteases e lipases não permite a separação completa do material fibroso de lipídeos e proteínas. Redondo-Cuenca; Villanueva-Suárez; Mateos-Aparicio (2008) detectaram 72% de eficiência na remoção de proteínas em amostras de okara submetidas a determinação de fibra pelo método AOAC.

Independente da solubilidade, a liberação de proteína requer a ruptura da parede celular dos cotilédones da soja (CAMPBELL *et al*., 2011). Rosenthal; Pyle; Niranjan (1998) apresentaram um modelo de rendimento de extração de óleo e de proteína baseado no princípio de rompimento de células na superfície das partículas de farinha que permite a liberação destes componentes, estabelecendo que o rendimento é diretamente proporcional ao inverso do tamanho da partícula. Os autores explicam que, uma vez que as células estejam rompidas, a água pode penetrar facilmente e as espécies solúveis e insolúveis associadas às células podem ser liberadas para o ambiente e que os rendimentos na extração de proteína e lipídeo são proporcionais às células rompidas. A liberação destes componentes ocorre provavelmente devido ao menor comprimento do percurso de difusão oferecido pela farinha fina, o que permite liberação quase instantânea da proteína solubilizada das células quebradas. O rompimento de parede celular acelera a transferência de massa e a cinética de extração (JUNG; MAHFUZ, 2009) e a agitação aumenta o rendimento de extração porque causa ruptura adicional (ROSENTHAL *et al*., 1998).

A tecnologia de alta pressão tem sido utilizada para manter ou melhorar características sensoriais e funcionais de alimentos (MOR-MUR, 2010; PORFIRI *et al*., 2017; YORDANOV; ANGELOVA, 2010), inclusive em leguminosas (ESTRADA-GIRÓN *et al*., 2005). A HAP pode alterar o tamanho e a área de superficial das partículas (HUANG *et al*., 2015) o que pode, por exemplo, influenciar a capacidade de extração de proteína da soja e do okara (VISHWANATHAN *et al*., 2011).

Omi et al. (1996) estudaram o uso de alta pressão isostática (100-700 MPa/20°C/25 minutos) para liberar proteínas de grãos de soja e também observaram aumento da extração com aumento da pressão até 400 MPa. Porfiri *et al*. (2017) utilizaram a homogeneização a alta pressão (100 MPa, 3 passagens) como pré-tratamento de okara a ser submetido a autoclavagem para produção de polissacarídeos insolúveis de soja. Os resultados da pesquisa mostraram que a HAP aumentou a concentração de componentes solúveis (polissacarídeos e peptídeos) em meio ácido durante a autoclavagem. Esta constatação pode ser relacionada com o enfraquecimento ou ruptura de ligações não covalentes entre polissacarídeos e agregados proteicos pela alta pressão e exposição das estruturas internas destas moléculas.

A capacidade de retenção de água (CRA) é também uma propriedade tecnológica importante relacionada à retenção de água dentro da microestrutura do alimento, o que a torna um atributo que pode ser utilizado para modificar a textura (FUNG; YUEN; LIONG, 2010) e o redimento do alimento (LI *et al*., 2015). CRA refere-se à habilidade de absorver a água e retê-la contra a força gravitacional dentro de uma matriz (DAMODARAN; PARKIN; FENNEMA, 2010). Esta capacidade está relacionada à formação de ligações de hidrogênio com moléculas de água (FUNG; YUEN; LIONG, 2010). Okara possuiu alta capacidade de se ligar à água (JANKOWIAK *et al*., 2014; NAKORNPANOM, HONGSPRABHAS; HONGSPRABHAS, 2010) devido as suas frações proteica e fibrosa (O'TOOLE, 1999; PALERMO; FIORE; FOGLIANO, 2012; SUREL; COUPLET, 2005). Em geral, a redução de tamanho de partícula ocasiona melhoria da CRA (MATEOS-APARICIO; MATEOS-PEINADO; RUPÉREZ, 2010; ULBRICH; FLOTER, 2014), inclusive em okara (FUNG *et al*., 2010; LI *et al*., 2013;).

2.3 Homogeneização a alta pressão

O termo "homogeneização" refere-se à capacidade de produzir uma distribuição de tamanho das partículas homogênea suspensas em um líquido que, sob o efeito de pressão, foi submetido a travessia por uma válvula de homogeneização (PATRIGNANI; LANCIOTTI, 2016). O propósito é obter propriedades macroscópicas uniformes por meio de uma redistribuição espacial de materiais (MARTÍNEZ-MONTEAGUDO; YAN, 2017). Na indústria de alimentos são utilizadas a homogeneização convencional (HC) e a homogeneização a alta pressão (HAP), sendo a primeira uma operação unitária tradicionalmente utilizada com o objetivo de misturar, dispersar, emulsificar ou encapsular os componentes de um sistema (PALMERO *et al*., 2016; PATRIGNANI; LANCIOTTI, 2016; WEI *et al*., 2018; ZHU *et al*., 2016). A HAP, também denominada homogeneização a alta pressão dinâmica ou

homogeneização a ultra-alta pressão (ALIA, *et al*., 2018) é uma tecnologia de processamento a AP baseada nos princípios da homogeneização convencional, só que em níveis de pressão mais elevados (MOR-MUR, 2010), superiores a 60 MPa (DUMAY *et al*., 2013; MARTÍNEZ-MONTEAGUDO; YAN, 2017).

A AP é uma tecnologia que tem sido utilizada para conservar e transformar alimentos, promovendo a obtenção de produtos seguros, mas que mantém ou melhoram as suas características sensoriais e nutricionais (MOR-MUR, 2010). Pode ser utilizada para inativar enzimas e micro-organismos (YORDANOV; ANGELOVA, 2010); inativar fatores antinutricionais (GUERRERO-BELTRÁN *et al*., 2009); reduzir off-flavor e melhorar de propriedades tecnológicas em leguminosas (ESTRADA-GIRÓN; SWANSON; BARBOSA-CÁNOVAS, 2005); dissociar proteínas oligoméricas e complexos proteicos (Mor-Mur, 2010), modificar a digestibilidade de proteínas (ESTRADA-GIRÓN *et al*., 2005); controlar ou modificar as interações entre proteínas e polissacarídeos (Torrezan & Cristianini, 2005); alterar a estrutura de fibras dietéticas (TEJADA-ORTIGOZA *et al*., 2015), como por exemplo, com a transformação de fibra insolúvel em solúvel (HUANG *et al*., 2015); melhorar a extração de componentes, como fibras dietéticas e proteínas (TEJADA-ORTIGOZA *et al*., 2015); alterar as propriedades sensoriais e a funcionalidade de vários componentes dos alimentos (YORDANOV; ANGELOVA, 2010), melhorando ou criando textura e aprimorando atributos tecnológicos, como as capacidades de retenção de água e óleo e de inchamento, por exemplo (HUANG *et al*., 2015; MATEOS-APARICIO; MATEOS-PEINADO; RUPÉREZ, 2010; TORREZAN; CRISTIANINI, 2005; ULBRICH; FLÖTER, 2014) e, consequentemente afetando propriedades macroscópicas dos alimentos como fluidez, estabilidade e textura (TORREZAN *et al*., 2007).

O processamento a AP pode ser conduzido com pressões de até 1400 MPa, com ou sem aquecimento, em alimentos líquidos ou em sólidos com alto teor de umidade (MOR-MUR, 2010; YORDANOV; ANGELOVA, 2010), empregando-se os sistemas isostático - Alta Pressão Isostática (API) ou dinâmico (HAP) (XIE *et al*., 2018). No sistema de API, o alimento sólido ou líquido, embalado em envólucro flexível, é colocado em uma câmara contendo um fluido (em geral, água) que transmite, igualmente e instantaneamente, a pressão ao alimento, geralmente até 600 MPa (XIE *et al*., 2018). Os princípios de *Le Chatelier* e o Isostático regem o comportamento dos alimentos submetidos a esta tecnologia (CAMPOS; DOSUALDO; CRISTIANINI, 2003; YORDANOV; ANGELOVA, 2010).

Na HAP, o alimento líquido pressurizado, em geral até 400 MPa (MARTÍNEZ-MONTEAGUDO; YAN, 2017), é forçado a passar por uma válvula com abertura estreita (Diagrama esquemático apresentado na Fig. 2.4). Há grande queda de pressão e aumento de velocidade, o que resulta em fenômenos de cisalhamento, turbulência, cavitação e fricção (ALIA *et al*., 2018; GUL *et al*., 2017; MOR-MUR, 2010; SARICAOGLU *et al*., 2017). Quando o fluido é forçado através do espaço da válvula de homogeneização muito estreita, partículas e macromoléculas em suspensão são submetidas a torções e deformações (FLOURY *et al*., 2004). Isto pode ocasionar significante redução do tamanho das partículas na faixa de microns ou submicrons (CHEN; XU; ZHOU, 2016), tornando-as mais uniformes (PALMERO *et al*., 2016). As pressões utilizadas em HAP (100-300 MPa) não são suficientes para garantir a segurança microbiológica do fluido, mas é possível obter efeito de pasteurização ou esterilização comercial manipulando vários fatores, como a temperatura de entrada do fluido (MARTÍNEZ-MONTEAGUDO; YAN, 2017).

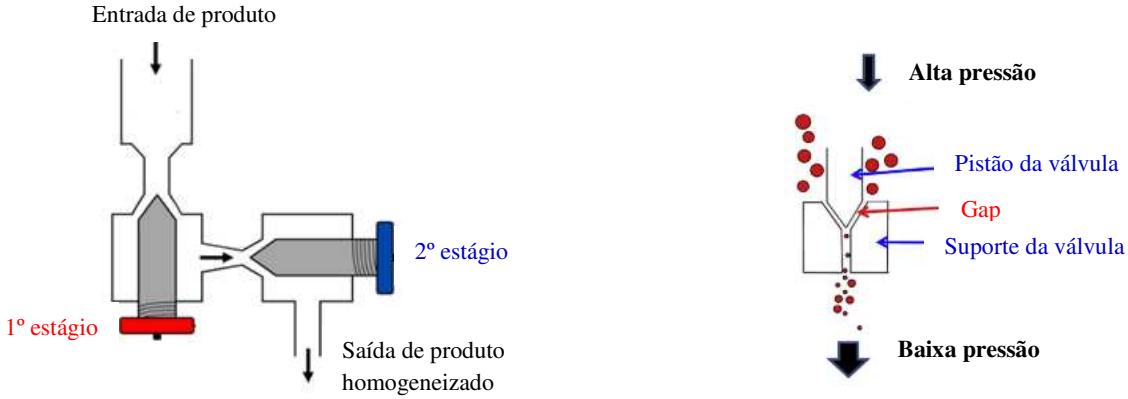

Fig. 2.4. Diagrama esquemático de um homogeneizador a alta pressão com 2 estágios e representação esquemática de duas válvulas de homogeneização.
Fonte: Adaptado de Dumay *et al*. (2013) e de Preece *et al*. (2017).

Embora ambos os sistemas sejam vantajosos por poderem ser utilizados como processamento não térmico (GUL *et al*., 2017) e por permitirem obter alimentos processados com poucas modificações em suas características nutricionais e sensoriais (YU *et al*., 2018), a HAP apresenta vantagens adicionais que são a elevada capacidade de processamento (MERCAN; SERT; AKIN, 2018) e a possibilidade de uso em sistema contínuo (CHEN; XU; ZHOU, 2016).

A HAP pode ser utilizada para romper estruturas celulares e seus fragmentos, ocasionando a inativação de micro-organismos ou promovendo a liberação de componentes

celulares ou aumentando o acesso do solvente a eles, o que pode melhorar a extração e o isolamento de seus componentes celulares (COMUZZO *et al*., 2017; WEI *et al*., 2018). Pode alterar atividade de enzima (PATRIGNANI; LANCIOTTI, 2016), aumentando-a ou diminuindo-a (MOR-MUR, 2010); afetar a conformação de proteína (YU *et al*., 2018) e clivar polissacarídeos (BERNAT *et al*., 2015; LEITE; AUGUSTO; CRISTIANINI, 2016), alterando a funcionalidade; reduzir a separação de fases, uniformizar a textura e melhorar as características reológicas (AUGUSTO; IBARZ; CRISTIANINI, 2013; AUGUSTO; IBARZ; CRISTIANINI, 2012; KUBO; AUGUSTO; CRISTIANINI, 2013; LEITE; AUGUSTO; CRISTIANINI, 2014, 2015, 2016; GUL *et al*., 2017; SARICAOGLU *et al*., 2017; YI *et al*., 2018).

A HAP tem mostrado ser uma tecnologia com grande potencial para fornecer produtos que atendam aos requerimentos dos consumidores por alimentos com a máxima retenção das propriedades nutricionais e sensoriais (PATRIGNANI; LANCIOTTI, 2016), mas ao mesmo tempo, esta tecnologia possibilita modificar os alimentos, o que pode ocasionar mudanças positivas que levarão a introdução de alimentos inovadores (CHEN; XU; ZHOU, 2016; PALMERO *et al*., 2016; ZHU *et al*., 2016). A redução de tamanho de partículas e outras possíveis alterações de componentes que a HAP pode promover, pode representar uma alternativa para melhorar a estrutura de okara, que por ser particulada, é descrita como um entrave ao seu aproveitamento (KASAI *et al*., 2004). Okara possui partículas com tamanhos entre 100 e 2000 μm, valores muito superiores aos 25 μm que são descritos como o menor tamanho detectável pelo palato, embora além de tamanho, a forma, a concentração de partículas e o meio em que as partículas estejam dispersas também sejam fatores que influenciam a percepção de textura (ENGELEN; VAN DER BILT, 2008). Além de poder melhorar a percepção sensorial devido ao aspecto de textura apontado, a HAP poderá promover e padronizar modificações desejáveis nas propriedades reológicas de okara, melhorando a sua consistência, o que poderá contribuir com sua estabilidade e facilitará a sua incorporação e manipulação na indústria, como no processo de desidratação, por exemplo.

Existem estudos com o uso de AP em sistemas a base de soja, com diferentes objetivos, como por exemplo, para melhorar a formação de geis (ALVAREZ; RAMASWAMY; ISMAIL, 2008; APICHARTSRANGKOON, 2003; CRUZ *et al*., 2009; HUANG; KUO, 2015; LAMBALLERIE, 2010; LIU; CHIEN; & KUO, 2013; SPERONI; JUNG; MOLINA; DEFAYE; LEDWARD, 2002; SONG *et al*., 2013; SPERONI *et al*., 2009); a hidrólise enzimática de proteínas (GIRGH *et al*., 2015; PEÑAS *et al*., 2006; PEÑAS; PRÉSTAMO;

GÓMEZ, 2004, PEÑAS; PRÉSTAMO; GOMEZ, 2004); inativar inibidores de tripsina (GUERRERO-BELTRÁN et al., 2009); alterar o perfil de fibras (MATEOS-APARICIO *et al*., 2010; PÉREZ-LÓPEZ *et al*., 2016; TU *et al*., 2012); alterar proteínas (AÑÓN; LAMBALLERIE; SPERONI, 2012; AÑÓN; LAMBALLERIE; SPERONI, 2011; LI *et al*., 2012; LIU & KUO, 2016; OMI *et al*., 1996; PEÑAS *et al*., 2011; PEÑAS *et al*., 2006; PUPPO *et al*., 2011; PUPPO *et al*., 2005; ZHANG; LITE; MITTAL, 2010); melhorar propriedades funcionais (FERNÁNDEZ-ÁVILA; ESCRIU; TORREZAN, *et al*., 2007; TRUJILLO, 2015); melhorar a extração de componentes biotivos (JUNG; MURPHY; SALA, 2008; TORO-FUNES *et al*., 2014); e melhorar a estabilidade microbiana e produção de emulsões (CRUZ *et al*., 2007; FLOURY; DESRUMAUX; LEGRAND, 2002; POLISELI-SCOPEL *et al*., 2014; PRÉSTAMO *et al*., 2000; SMITH; MENDONCA; JUNG, 2009).

Especificamente aplicando HAP em okara, o trabalho de Preece *et al*. (2017) teve o propósito de aumentar o rendimento de extração de proteínas, lipídeos e sólidos totais em okara utilizando pressões de 50 a 125 MPa. No entanto, não encontramos na literatura nenhum trabalho que investigasse efeitos da HAP com o objetivo de melhorar as suas características físicas, o que poderia facilitar a sua incorporação em sistemas alimentícios ou o seu processamento em outras etapas de transformação industrial.

2.4 Digestão gastrointestinal e microestrutura do alimento

Okara é um resíduo barato, com relevante quantidade de proteínas e que contém todos os aminoácidos essenciais (O'TOOLE, 1999), e por isso é considerado uma fonte potencial de proteína de baixo custo para nutrição humana (TAMURA *et al*., 1999). Entretanto, embora a composição de aminoácidos possa indicar a qualidade das proteínas, para que um alimento seja considerado boa fonte deste nutriente na alimentação e a sua utilização seja incentivada, é necessário que se avalie outros fatores, como por exemplo, a proporção entre os aminoácidos e a disponibilidade deles, sendo que a disponibilidade depende de vários fatores como tratamentos prévios que a proteína passou no processamento, interações com outros componentes dos alimentos, sua digestibilidade, absorção e utilização no corpo humano (VAGADIA; VANG; RAGHAVAN, 2017). Inicialmente, a utilização dos aminoácidos pelo organismo dependerá da hidrólise da cadeia polipeptídica pelas enzimas proteolíticas durante a digestão (COZZOLINO, 2012).

A digestão humana é um processo complexo que gera nutrientes individuais que poderão ser absorvidos através da parede do trato gastrointestinal (SINGH; GALLIER, 2014).

Durante a digestão ocorrem, simultaneamente, dois processos principais que são: a redução do tamanho das partículas e as transformações enzimáticas, relacionadas a hidrólise de macromoléculas em constituintes que podem ser absorvidos pela corrente sanguínea. A Fig. 2.5 apresenta um resumo das etapas de digestão, que se inicia na boca com a mastigação e tem atuação das enzimas salivares amilase e lipase, por curto intervalo de tempo. O bolo alimentar resultante é transportado por mecanismo de peristaltismo através do esôfago para o estômago, onde ondas peristálticas ajudam a quebrar o alimento e em contato com o suco gástrico (que é composto por ácido clorídrico e pelas enzimas pepsina e lipase) ocorre a digestão de proteínas e lipídeos. O quimo resultante segue para o intestino delgado, especificamente para o duodeno, região onde é neutralizado com bicarbonato de sódio, os lipídeos são emulsificados com ação da bile produzida pelo fígado e os nutrientes são hidrolisados pelas enzimas pancreáticas (proteases, amilases e lipases) e por outras enzimas da parede do intestino. Água e nutrientes são absorvidos pelas enterócitos nas vilosidades e o material não absorvido segue para o intestino grosso, onde ocorre a absorção de água e de eletrólitos, a fermentação de polissacarídeos e de proteínas por micro-organismos, a reabsorção de sais biliares e a formação, armazenamento e eliminação de fezes (GUERRA *et al*., 2012).

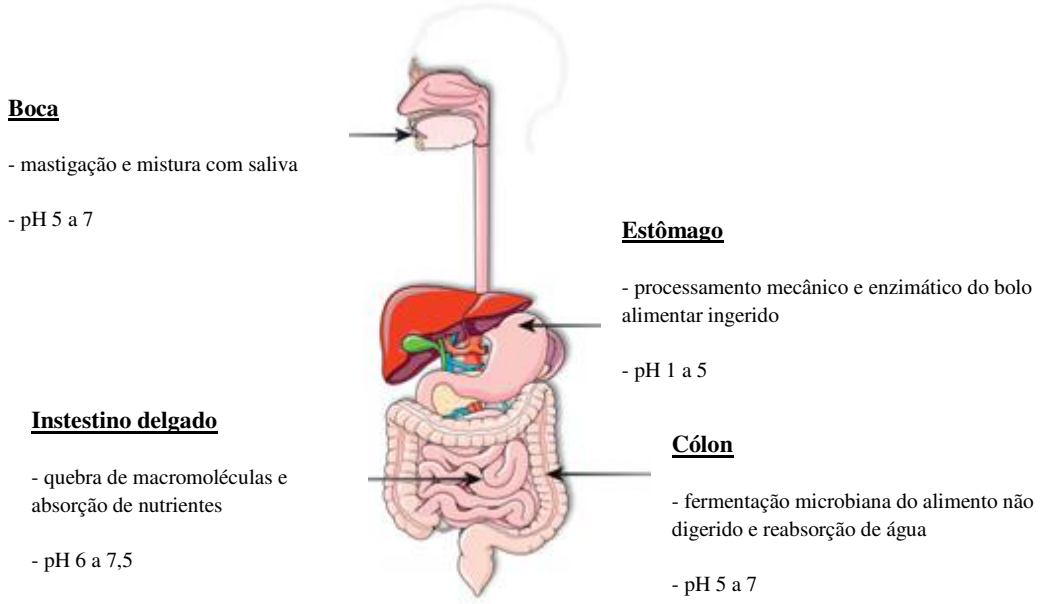

Fig. 2.5. Representação das regiões do trato gastrointestinal humano e das fases da digestão. Fonte: Adaptado de Guerra *et al*. (2012).

A estrutura do alimento pode facilitar ou limitar a liberação dos nutrientes que se tornarão disponíveis para serem absorvidos pelo organismo – bioacessíveis (MAT *et al*., 2016;

MORENO; MACKIE; MILLS, 2005). A bioacessibilidade é importante condição para que os nutrientes absorvidos possam estar disponíveis para o organismo nas suas funções fisiológicas (Fig. 2.6.). Além da ação mecânica e da atuação de ácidos e enzimas no processo digestório, as técnicas de processamento industrial às quais o alimento é submetido podem tornar os nutrientes bioacessíveis. Esta constatação é especialmente relevante quando se trata de vegetais, que possuem parede celular que limita a bioacessibilidade de nutrientes (SINGH; GALLIER, 2014).

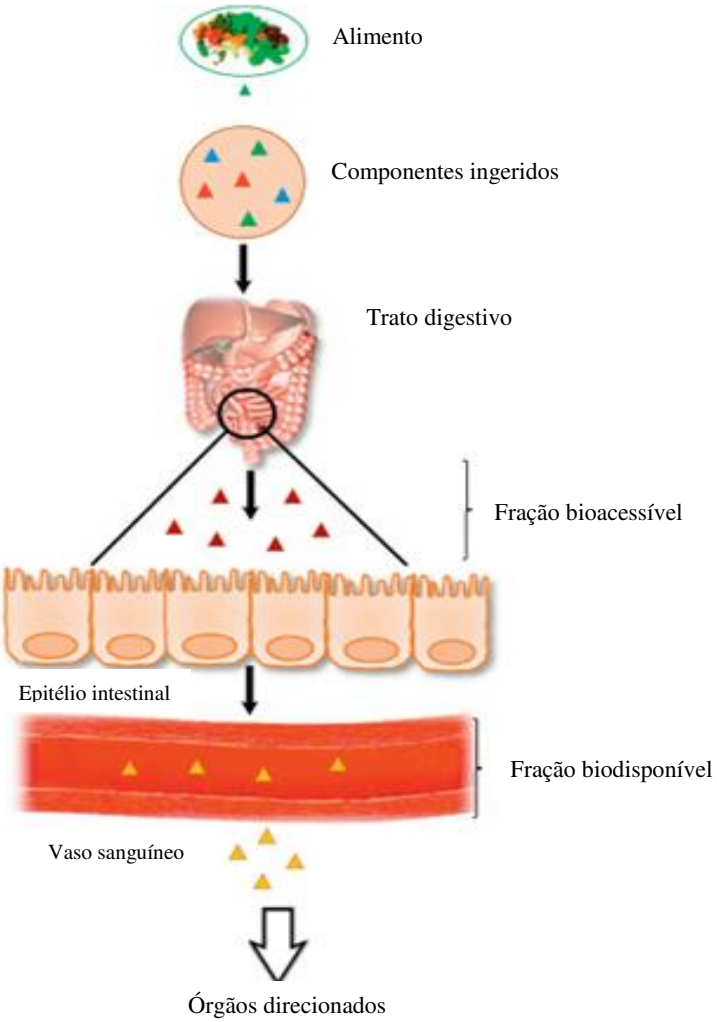

Fig. 2.6. Representação das frações bioacessíveis e biodisponíveis de componentes ingeridos e submetidos a digestão gastrointestinal humana.
Fonte: Adaptado de Guerra *et al*. (2012).

Experimentos *in vitro* e *in vivo* têm mostrado que a estrutura dos alimentos à base de plantas pode ter um efeito importante na digestão dos macronutrientes alimentares, como por exemplo, proteínas encapsuladas dentro de paredes celulares não são hidrolisadas pelas enzimas digestivas humanas (BHATTARAI *et al*., 2018). A liberação de proteínas durante a digestão é dependente da sua estrutura e de múltiplas interações naturais que elas estabelecem na matriz complexa do alimento, de outras que podem ser desencadeadas pelo processamento, além daquelas passíveis de ocorrer com outros micro ou macronutrientes no trato gastrointestinal. Dependendo da fonte e do processamento, as proteínas podem exibir várias estruturas heterogêneas e complexas que podem influenciar a acessibilidade a enzimas proteolíticas do trato intestinal (SINGH; GALLIER, 2014). Além disso, fibras e outros polissacarídeos podem afetar a digestão de proteínas (FAFAUNGWITHAYAKUL; HONGSPRABHAS; HONGSPRABHAS, 2011; TURGEON, RIOUX, 2012), pois estes podem impedir acesso de proteases digestivas aos locais de clivagem nas proteínas e peptídeos via interações eletrostáticas ou devido ao aumento da viscosidade do conteúdo gastrointestinal (SINGH; GALLIER, 2014).

Bhattarai *et al*. (2018) estudaram como a estrutura celular intacta contribui com a susceptibilidade às enzimas. Inicialmente, os autores mostraram que células de leguminosas são mais resistentes a desintegração do que a de cereais por processo mecânico, pois enquanto as primeiras necessitavam de 16 horas para serem quebradas, as segundas requeriam 6 horas. Os autores constataram que células intactas são resistentes à α-amilase, pois a taxa e extensão da hidrólise do amido eram menores neste caso, quando comparadas às de células quebradas e da farinha. Esta pesquisa concluiu que a parede celular além de atuar como barreira na difusão da enzima, também serviu como superfície adsorvente, pois a enzima ficou predominantemente ligada à superfície externa das células, o que indicou que havia afinidade tanto por substratos catalíticos (amido) como também por não-catalíticos (parede celular).

Bhattarai *et al*. (2017), ao avaliarem a hidrólise enzimática de amido e proteínas em células de leguminosas (grão de bico, ervilha e feijão), constataram que as taxas de hidrólise de proteína e amido eram 20 vezes maiores quando a estrutura celular era quebrada em comparação com as células intactas e, que o modelo de digestão estudado era incapaz de romper as estruturas intactas. Os autores concluíram que a estrutura celular intacta funciona como uma barreira que controla a difusão da enzima no interior das células o que provavelmente limita a taxa de hidrólise. A pesquisa também mostrou que a adição de fibras isoladas de parede celular de

leguminosas diminuiu a hidrólise. Estes resultados sugeriram que interações estruturais entre macromoléculas afetaram a suscetibilidade às enzimas.

A organização da matriz e o processamento poderão ter efeito positivo ou negativo na digestão de nutrientes (MAT *et al*., 2016; VALLE; SOUCHON; ANTON, 2013; TURGEON; RIOUX, 2011). Estrada-Girón *et al*. (2005) destacam que a digestibilidade de proteínas pode ser modificada pela tecnologia de alta pressão. O processamento por HAP, por exemplo, pode expor proteínas encapsuladas dentro de paredes celulares (BHATTARAI *et al*., 2018; SINGH; GALLIER, 2014), romper interações entre proteínas e outros componentes como fibras e outros polissacarídeos (FAFAUNGWITHAYAKUL; HONGSPRABHAS; HONGSPRABHAS, 2011; TURGEON; RIOUX, 2011), e, em consequência, facilitar a hidrólise pelas enzimas digestivas, aumentando, então, sua bioacessibilidade.

No caso de okara, autores têm relatado que as suas proteínas são parcialmente resistentes à digestão gastrointestinal com pepsina e pancreatina (VONG; LIU, 2016). Jiménez-Escrig *et al*. (2010) simularam a digestão *in vitro* de isolado proteico de okara e constataram grau de hidrólise de 53%, o que demonstrou que as proteínas de okara resistiram parcialmente a digestão por pepsina e pancreatina. Mesmo com a parede celular rompida, a digestão incompleta das proteínas de okara foi observada (VONG; LIU, 2016; JIMÉNEZ-ESCRIG *et al*., 2010).

Os estudos conduzidos para obter informações sobre as alterações que os componentes dos alimentos passam no processo digestório são geralmente realizados com ensaios *in vitro*, que são alternativas rápidas, menos dispendiosas e trabalhosas e não envolvem restrições éticas como no caso de testes *in vivo* (MINEKUS *et al*., 2014). Mas é importante destacar que diferentes dos modelos *in vivo*, as simulações *in vitro* são simplificados e não consideram a microbiota residente, o sistema imunológico e os controles hormonais específicos, por exemplo. Modelos estáticos não consideram ainda parâmetros como taxa de esvaziamento gástrico e tempo de trânsito intestinal, que podem influenciar a bioacessibilidade de componentes, pois impactam na liberação dos mesmos da matriz, na sua solubilidade e estabilidade (GUERRA *et al*., 2012).

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CAPÍTULO 3: ARTIGO 1 - EFFECT OF HIGH-PRESSURE HOMOGENIZATION ON THE PHYSICAL PROPERTIES OF AN AQUEOUS DISPERSION OF OKARA

Artigo submetido ao Food Research International.

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ABSTRACT

High-pressure homogenization (HPH) was used to modify the physical characteristics of okara. An aqueous dispersion of okara was subjected to HPH (up to 175 MPa) using one or multiple passages (2 or 3 passages). HPH modified the appearance and the color of okara, ΔE* increased up to 5 times with one passage through the homogenizer. The use of one passage led to a reduction of 82%, 92%, 66%, and 85% of D[3,2], D[4,3], yield stress (σ0), and consistency index (k), respectively, and an increase in flow behavior index (n) up to 53%. The multiple passages affected the rheological parameters mainly at lower pressures and up to two passages. Microscopic observations revealed cell disruption and changes in the microstructure of okara subjected to HPH. The extractable protein increased with the HPH. The effects of HPH on okara characteristics, especially the decrease in consistency, may be due to the reduction of particle size and the increase in extractability protein, suggesting that this technology can provide quality characteristics which make okara more suitable for use in food systems or to be handled or processed industrially.

Keywords: dynamic high-pressure, microstructure, rheology, soybean agro-waste.

3.1 Introduction

The production of water-soluble soybean extracts (WSSE) generates a by-product known as okara (Li, Qiao, & Lu, 2012) which contains, on average, 42.5% fibers, 37% proteins, 13% lipids, 4.7% soluble carbohydrates, and 2.8% ash (on a dry basis) (Bowles & Demiate, 2006). Vitamins and phytochemicals such as phenolic compounds, coumestans, and anti-nutritional factors are also found in okara (Bowles & Demiate, 2006; Jiménez-Escrig, Tenorio, Espinosa-Martos, & Rupérez, 2008; Ohno, Ano, & Shoda, 1996; Quitain, Oro, Katoh, & Moriyoshi, 2006; Stanojevic, Barac, Pesic, Jakovic, &Vucelic-Radovic, 2013). Okara proteins have good nutritional and technological quality (Ma, Liu, Kwokb, & Keokb, 1997; Puechkamut & Panyathitipong, 2012). Okara carbohydrates are comprised of about 85% insoluble fiber and 7% soluble fiber, less than 1% starch, and about 7% low molecular weight carbohydrates such as inulin, stachyose, raffinose, sucrose, glucose, galactose, arabinose, and fructose (Mateos-Aparicio, Redondo-Cuenca, Villanueva-Suárez, Zapata-Revilla, & Tenorio-Sanz, 2010).

The relevant nutritional quality and bioactive potential of okara have led to investigations about the use of this by-product in food formulations (Cervantes, Aoki, Almeida, Nepomuceno, & Pulzatoo, 2010; Li, Lu, Nan, & Liu, 2012). Studies have shown that okara is a potential bioactive food ingredient as it is composed mainly of fibers and proteins, which have hypocholesterolemic, hypoglycemic, antihypertensive, and antioxidant effect (Amin & Mukhrizah, 2006; Jiménez-Escrig et al., 2008; Li, Qiao & Lu, 2012; O'Toole, 1999; Villanueva, Yokoyama, Hong, Barttley, & Rupérez, 2011). However, okara consists of a particulate heterogeneous material with a high cohesiveness, which impairs its use in liquid systems or dehydration using conventional methods (Taruna & Jindal, 2002; Wei, Ye, Li, Wang, Li, & Zhao, 2018).

High-pressure homogenization (HPH) technology can be used to ensure the safety and quality of food products (Mor-Mur, 2010). HPH has been used to change the activity of

enzymes and microorganisms and to alter the sensory and functional properties of various food components (Yordanov & Angelova, 2010), including new texture profiles (Messens, Camp, & Huyghebaert, 1997; Torrezan & Cristianini, 2005). HPH can also inactivate antinutritional factors (Guerrero-Beltrán, Estrada-Girón, Swanson, & Barbosa-Cánovas, 2009), reduce protein allergenicity (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011) and off-flavors, and improve the technological properties of legumes (Estrada-Girón, Swanson, & Barbosa-Cánovas, 2005).

Thus, considering that HPH is a promising technology to alter the rheological properties of foods, and okara is an underutilized waste of the food industry, the objective of this study was to investigate the effect of HPH and the use of multiple passages (MP) on the physical characteristics of aqueous dispersion of okara.

3.2 Material and methods

3.2.1 Obtaining and characterizing okara

Soybean (*Glycine max L. Merril*) variety IAC Foscarin 31 was purchased from the Agronomic Institute of Campinas (Campinas, SP, Brazil). Okara was obtained in the laboratory, as reported by Benedetti & Falcão (2003) for the production of WSSE, with adaptations. The processing conditions were similar to those used on an industrial scale. Soybeans were washed in running water, immersed in water (1:7 w/v) for 30 minutes at room temperature, drained, manually peeled, and maintained in a hot water bath (1:7 w/v; 97 ºC) for 14 minutes. The grains were drained, milled in an industrial blender for 5 minutes using water at 60 ºC (1:7 w/v), and filtered through a synthetic fabric to obtain okara and WSSE. Soybeans and okara were characterized for moisture, protein, ash (AOAC, 2000), lipids (AOAC, 2006), and total

carbohydrates (calculated by difference). Okara was vacuum-packed and stored at -18 °C until use.

3.2.2 High-pressure homogenization (HPH)

Prior to the assays, okara was thawed and diluted in water to reach a protein content of 5% (w/v), which was established in preliminary tests as the maximum adequate concentration to be processed in the homogenizer. The aqueous dispersion of okara was pre-processed in a high shear mixer (Ultra Turrax T-18 Basic, IKA Labortecnik, Wilmington, NC) at 24,000 rpm/min for 5 minutes and subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, 2, or 3) using a Panda Plus 2000 homogenizer (GEA NiroSoavi, Italy) at a flow rate of 9 L/h. The homogenizer was fed with the samples at 20 ± 5 °C, and the processed material was immediately cooled in an ice bath at the same temperature condition. An increase in temperature was observed with the increase in pressure conditions, with values of 19 ± 3 °C; 20 ± 3 °C; 24 ± 3 °C; 26 ± 3 °C; and 37 ± 3 °C for 0, 25, 75, 125, and 175 MPa, respectively. The samples subjected to multiple passages were cooled to 20 ± 5 °C before the next pass through the homogenizer. Samples were stored at 7 ± 3 °C until analysis (maximum 24 hours). The experiment was carried out in three replicates, with two controls consisting of an aqueous dispersion of okara standardized to 5% (w/v) protein, with (OCT) or without pre-processing (OST). The samples were identified as abbreviations referring to the pressure and number of passages, as follows: 0 MPa-1p, 0 MPa-2p, 0 MPa-3p, 25 MPa-1p, 25 MPa-2p, 25 MPa-3p, 75 MPa-1p, 75 MPa-2p, 75 MPa-3p, 125 MPa-1p, 125 MPa-2p, 125 MPa-3p, 175 MPa-1p, 175 MPa-2p, and 175 MPa-3p.

3.2.3 Particle size distribution

The particle size distribution was determined in a Master sizer 2000 apparatus (Malvern Instruments, Delft, Worcestershire, UK). For that, refractive indices of 1.45 and 1.33 were used for water and the particles, respectively (Preece, Drost, Hooshyar, Krijgsman, Cox, & Zuidam, 2015; Preece, Hossyarb, Frijgsman, Fryera, & Zuidam, 2017). The samples were diluted with deionized water in the measurement cell until approximately 10% of laser obscuration was reached. The mean particle diameter was measured according to area D [3,2] and volume D [4,3]. The mean diameter and size distribution were calculated as the average of three replicates, in triplicate.

3.2.4 Rheological properties

The rheological properties were determined according to Leite, Augusto, & Cristianini (2014) in a controlled stress (σ) rheometer AR2000ex (TA Instruments, Denver, USA) at a constant temperature of 25 °C, controlled by a Peltier system. The rheometer was equipped with cross-hatched parallel plates (40 mm diameter) and gap adjusted to 1,200 μm obtained by a preliminary test. The samples were kept at rest for 5 min and then subjected to a constant shear rate at 300 s^{-1} for 300 s to determine thixotropic (time-dependent) behavior. For the study of fluid flow behavior, a protocol with a gradual linear reduction (300 s^{-1} to 0.1 s^{-1}) was used to guarantee the conditions required to evaluate the steady-state behavior and the flow curves (shear stress x shear rate) were determined. The results were modeled using Herschel-Bulkley's model (Eq. 1).

$$\sigma=\sigma_0+k\cdot\dot{\gamma}^n \quad (1)$$

where σ0 the yield stress (Pa), which represents the minimum shear stress to initiate flow; k is the consistency index (Pa) during the shear stress reduction; $\dot{\gamma}$ is the shear rate (s^{-1}), and n is the

flow behavior index (non-dimensional). The parameters of each model were obtained by non-linear regression using Curve Expert Professional 2.2.0 software (CurveExpert, USA) at the 95 % probability level.

3.2.5 Confocal laser scanning microscopy

The microstructure of the samples was analyzed by confocal laser scanning microscopy, according to the methodology described by Liu et al. (2019) with some adaptations. Protein, and lipids arrangements were observed in Axiovert200 M microscope with LSM 510 scanning module equipped with an argon laser (458, 477, 488, and 514 nm) and helium-neon (543 and 633 nm). Fast green (FGF) and Nile red (NR) were used to dye proteins and lipids, respectively. The samples were prepared in microtubes as follow: 500 μLsample + 25 μL FGF (0.1% w/v) + 25 μL NR (0.234% w/v). The samples were agitated for 1 minute, left to rest for 5 minutes, and transferred to slides with a 10 mm depth cavity. The observations were performed using an oil-immersion objective (63x/1.4 NA/oil) and the images were taken at a resolution of 1024 x 1024 pixels. The stained samples were visualized using excitation wavelengths of 633 and 488 nm (He/Ne laser), and emission wavelengths of 650 nm and 505-550 nm, for the observation of protein (red) and lipid (green) particles.

3.2.6 Color measurements

The instrumental color of the samples was measured using an ULTRA PRO colorimeter (Hunter Lab®, D65, Hunter Associates Laboratory, Virginia, USA), in reflectance mode with D65 illuminant and 10° observer angle (Rodrigo, van Loey, & Hendrickx, 2007; Sánchez-Moreno, Plaza, de Ancos & Cano, 2006). The measurement was performed in a quartz cuvette with an optical path length of 10 mm (20 mL), in triplicate. The color of each sample was determined using the CIELab parameters L* (lightness, black to white), and a* and b*

coordinates (green to red, and blue to yellow, respectively). The total color difference (ΔE*) and the whiteness indices (WI) were determined according to Eq. 2 (Choi, Kim, & Lee, 2002) and Eq.3 (Bernat, Chafer, Rodriguez-Garcia, Chiralt, & Gonzalez-Martinez, 2015), respectively.

$$\Delta E^* = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{\frac{1}{2}} \quad (2)$$

$$WI = 100 - ((100-L^*)^2 + (a^*)^2 + (b^*)^2)^{\frac{1}{2}} \quad (3)$$

3.2.7 Total, insoluble, and soluble fiber

The total, insoluble, and soluble fiber contents were determined for the samples 25-1p, 25 MPa-2p, 125-1p, and 175 MPa-1p, according to AOAC (2012).

3.2.8 Extractable proteins

The determination of extractable proteins was performed according to Morr et al. (1985). Briefly, 15 g of okara was dispersed in 40 ml of distilled water and kept under magnetic stirring for 1 hour at room temperature. The dispersion was transferred to a 50 mL volumetric flask, the volume was completed with distilled water and centrifuged (20,000xg for 30 min at 4 ° C). The total nitrogen content was determined in the aqueous extracts by micro-Kjeldhal (AOAC, 2000) to determine the protein concentration (mg/mL). Protein extractability was calculated according to Eq. 4.

$$\text{extractable proteins (\%)} = \frac{(A \times 50)}{W \times (\frac{S}{100})} \times 100 \quad (4)$$

where A is the protein concentration of the supernatant (mg/mL); W is the sample mass (mg) and S is the protein concentration of the sample (%).

3.2.9 Statistical analysis

All analyses were performed in triplicate. Data normality was analyzed by the Kolmogorov-Smirnov test. The t-test, analysis of variance (ANOVA) and Tukey's test was used when necessary, at a significance level of $P < 0.05$ using the statistical package GraphPad Prism version 6.0 (GraphPad Software Inc., Califórnia, EUA).

3.3 Results and discussion

3.3.1 Characterization of soybeans and of okara

The proximate composition of soybeans (Table 3.1) is in accordance with the values reported in the literature (Mext, 2015; Multiterno, Rodrigues, Lima, Ida, & Kurozawa, 2017; Stanojevic et al., 2013; Vong & Liu, 2016). According to Mext (2015), the average composition of whole Brazilian soybeans comprises 8.3 g of water, 33.6 g of protein, 30.7 g of carbohydrates, 17.3 g of total dietary fiber (1.0 g of soluble fiber and 16.3 g of insoluble fiber), 22.6 g of lipids, and 4.8 g of ash in 100 g of edible portion.

The composition of okara depends on the soybean cultivar and the extraction efficiency of WSSE process (O'Toole, 2004; O'Toole, 1999), once it is not possible to extract all the nutrients from soybeans, resulting in a residue rich in high-quality proteins, among other nutrients (Cervantes et al., 2010; Pinto & Castro, 2008).

In this study, the okara showed high protein and low total carbohydrate contents (Table 3.1), which was expected since it was obtained from dehulled beans. According to O'Toole (2004), soybean peeling can be adopted in WSSE guidelines, which implies an increase

in the protein content and reduction of the fiber content, slightly increasing the extraction yield and reducing the amount of okara produced (Preece, Hooshyar, & Zuidam, 2017).

Table 3.1 Proximate composition (dry basis; g/100 g) of soybeans IAC Foscarin 31 and okara obtained from the processing of water-soluble soy extract (WSSE).

	Moisture	Protein	Lipids	Ash	Total Carbohydrates*
Soybeans	10.5±0.2	38.1±1.1	21.2±0.8	4.8±0.0	35.9
Okara	73.9±1.1	47.9±0.7	21.8±1.0	2.7±0.0	27.6

Results are mean of three replicates ± standard deviation.

*Calculated by difference.

3.3.2 Effects of HPH on the physical characteristics of okara

The effects of the high shear stress associated with the single-pass HPH treatment on the particle size distribution (PSD) and the particle diameter of okara are shown in Fig. 3.1. The pre-treatment modified the particle size distribution from ~ 100 to 2000 (OST) to ~ 40 to 1300 μm (OCT) ($p<0.05$). The D [3,2] and D [4,3] values decreased 58 and 38% after the pre-treatment ($p <0.05$). The HPH processing led to changes in PSD and particle size, from almost monomodal in OCT and 0 MPa-1p to bimodal from 25 MPa-1p. The peaks became narrower, with an evident region of small particles with increasing the homogenization pressure (HP) up to 75 MPa-1p (Fig. 3.1A). Significant reductions in D [3,2] and D [4,3] values were observed up to 75MPa-1p and 125 MPa-1p, respectively ($p <0.05$), as compared to OCT. There was a reduction from 26 to 82% in D [3,2] values and 13 to 92% in D [4,3] values when comparing the HPH treated samples to OCT (Fig. 3.1B). The changes in particle diameters were more pronounced between 0 MPa-1p and 75 MPa-1p when comparing the condition 75 MPa-1p to

175 MPa-1p. The reductions in D [3,2] and D [4,3] values were more accentuated at 25 MPa-1p.

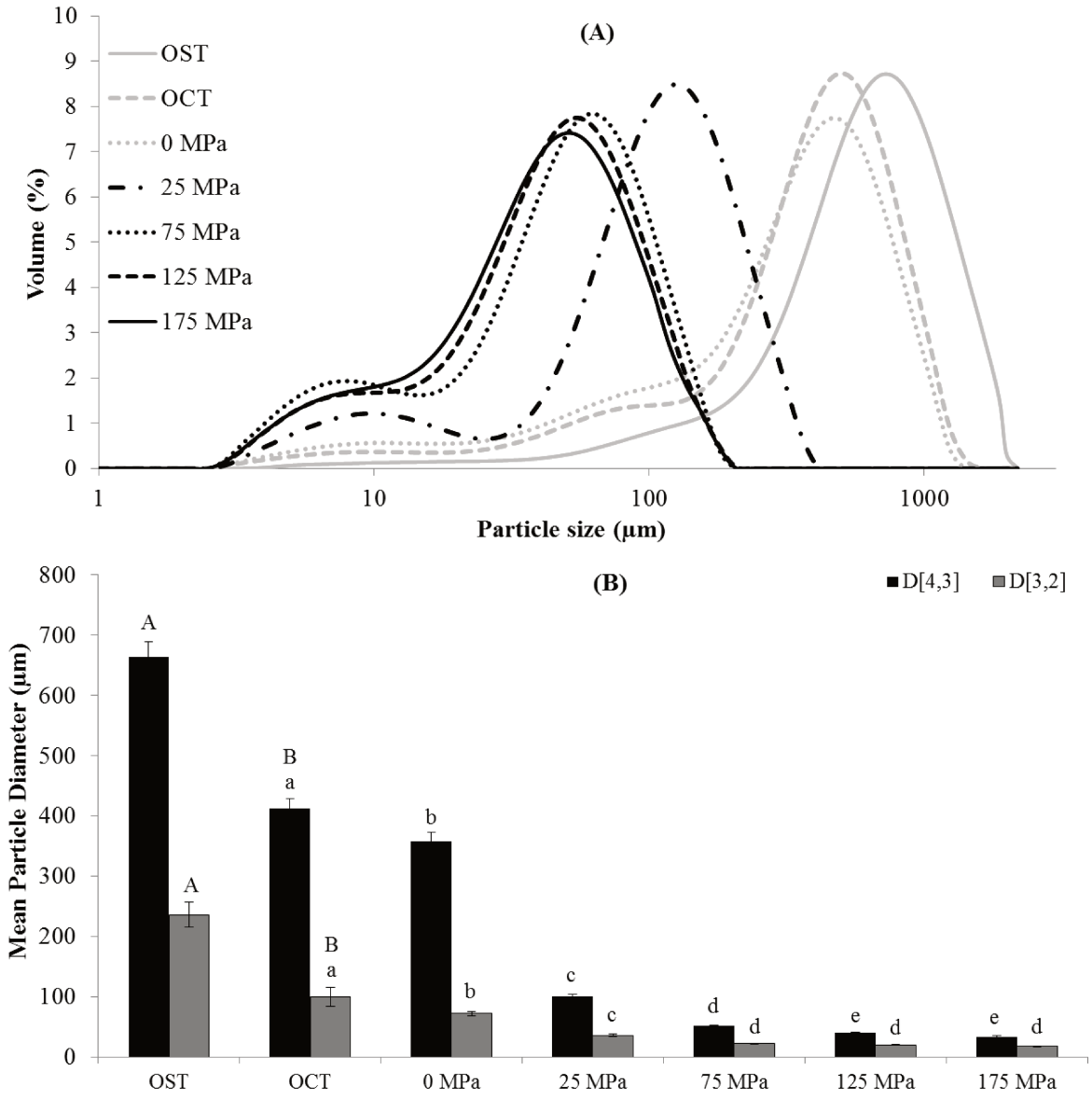

Fig. 3.1 Particle-size distribution (A) and particle diameter (B) of okara subjected to high-pressure homogenization (HPH), under different pressure conditions (0, 25, 75, 125, or 175 MPa). Means with same uppercase letters in columns of same color did not differentiate between them by the t-test ($p > 0.05$). The values D[4,3] or D[3,2] of OST and OCT were compared by t- test, and equal uppercase letters show no difference between them. The means

of all samples, except for OST, were compared using ANOVA and Tukey's test ($p > 0.05$); means with equal lowercase letters in columns of same color do not differentiate between them. Vertical lines represent the standard deviations. (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed).

The use of HPH with multiple passages (HPH-MP) is a strategy to obtain, at low pressures, the same effect of a simple passage at higher pressures (Leite, Augusto, & Cristianini, 2017). Considering these aspects, the effect of the multiple passages was evaluated in this research. The impact of 2 passages was more evident when compared to 3 passages (Fig. 3.2), and the span decreased with increasing the number of passages. No changes in the bimodal distribution were observed at 25 MPa as a function of the number of passages, while a nearly monomodal distribution was observed from 75 MPa. The homogenization at up to 125 MPa with 2 passages led to relevant and similar reductions of D [3,2] and D [4,3] values (~ 40%) ($p>0.05$), while increasing to 3 passages caused only a slight reduction of these parameters at 0 and 25 MPa (5-9%). There was no effect of the increase in the number of passages at 175 MPa ($p>0.05$). The use of 2 or 3 passages at the lower pressures (25 and 75 MPa) led to a reduction of particle size similar to that observed at higher pressures (125 e 175 MPa) using a single passage.

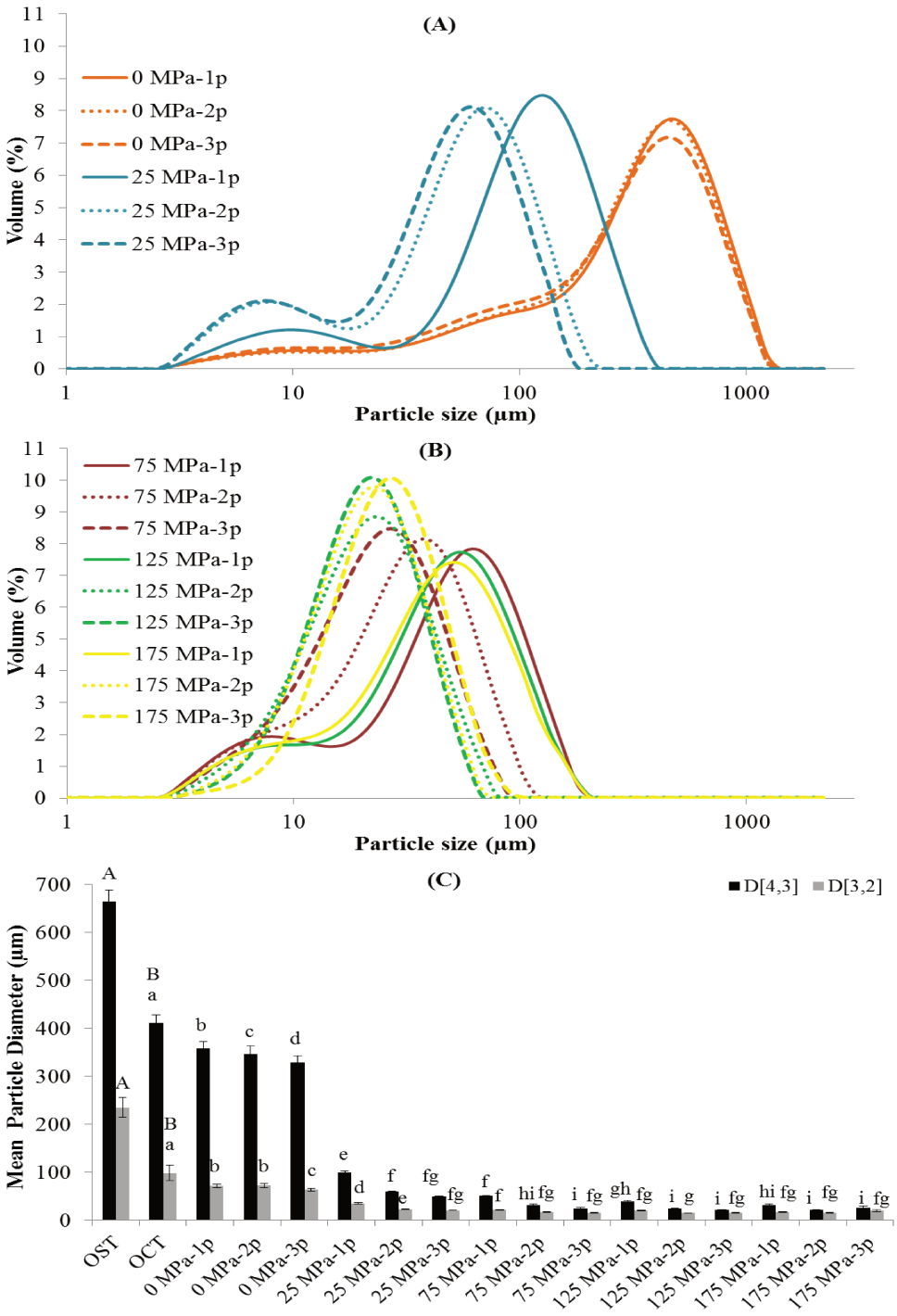

Fig. 3.2 Particle size distribution (3.2A, 3.2B) and particle size (3.2C) of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, 2, or 3). The results of OST and OCT were compared by t- test and equal uppercase letters show no difference between them. Means of all samples, except for OST, were compared using ANOVA and Tukey's test ($p > 0.05$); Means with equal

lowercase letters in columns of same color do not differentiate between them by the Tukey's test ($p > 0.05$). (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were pre-processed; 1p, 2p, and 3p correspond to the number of passages).

In non-homogenized samples, the particles size of the okara dispersion, from 100 to 2000 µm, were much larger than is indicated as the smallest size detectable by the palate, 25 µm (Engelen, Van Der Bilt, & 2008), which were reduced to 16 - 100 µm with the HPH from 25 MPa. Therefore, okara, which had initially a particulate structure described as a barrier to its exploitation (Kasai, Murata, Iniu, Sakamoto, & Kahn, 2004), may have its texture improved with the use of HPH. However, it should also be considered that the shape, particle concentration and the medium in which the particles are dispersed are also factors that influence the perception of texture (Engelen, Van Der Bilt, 2008).

3.3.3 Effects of HPH on the rheological properties of okara

The rheological behavior of the samples was described by the Herschel Bulkley (HB) model (Eq. 1), with excellent fit to the data ($R^2 > 0.98$), providing three rheological parameters as follows: yield stress ($\sigma 0$), consistency index (k), and flow behavior index (n).

The flow behavior and the effect of HPH using one passage, on the rheological parameters of okara, at 25 °C, are shown in Table 3.2 and Fig 3.3, and 3.4. The equation models of the parameters k (Eq. 5) and n (Eq. 6) as a function of pressure presented a good fit to the data ($R^2 > 0.98$). OST was not evaluated because it flowed out when subjected to the shear stress. When compared to OCT, the sample 25 MPa-1p showed a reduction of 66% and 85 % in $\sigma 0$ and k, and no further decrease was observed at pressures higher than 25 MPa-1p ($p > 0.05$). Regarding the parameter n, an increase of 45% and 53% was observed for 25 MPa-1p and 75 MPa-1p, respectively, when compared to OCT, with no more increment at pressures

higher than 75 MPa-1p ($p > 0.05$). The homogenization increased the flow behavior index and decreased the consistency index, as observed in other studies with plant matrices (Augusto, Ibarz, & Cristianini, 2012; Donsí, Esposito, Lenza, Senatore, & Ferrari, 2009; Floury, Dersumaux, Axelos, & Legrand, 2002; Lagoueyte & Paquin, 1998; Silva, Sato, Barbosa, Dacanal, Ciro-Velásquez, Cunha, 2010).

$$k=22.1024*e^{(-0.0691PH)} \qquad R^2=0.98888 \qquad (5)$$

$$n=0.3391+0.0607*PH^{(0.304)} \qquad R^2=0.99044 \qquad (6)$$

Table 3.2 Flow properties of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa): Herschel Bulkley parameter values at 25 ° C, modeled as a function of the homogenization pressure.

Treatment	σ_0 (Pa)	k ($Pa.s^{-1}$)	n
OCT	30.14 ± 7.95 (b)	25.74 ± 5.80 (a)	0.27 ± 0.07 (c)
0 MPa-1p	45.36 ± 11.33 (a)	22.12 ± 10.76 (a)	0.34 ± 0.10 (c)
25 MPa-1p	10.23 ± 2.06 (c)	3.78 ± 1.14 (b)	0.49 ± 0.03 (b)
75 MPa-1p	16.78 ± 0.67 (c)	1.63 ± 0.06 (b)	0.58 ± 0.01(a)
125 MPa-1p	12.94 ± 0.34 (c)	0.94 ± 0.03(b)	0.61 ± 0.01(a)
175 MPa-1p	13.25 ± 0.90 (c)	0.76 ± 0.04 (b)	0.62 ± 0.01(a)

Results are mean of triplicates ± standard deviation. Means with the same letters in the column do not differ statistically by the Tukey's test ($p > 0.05$). (OCT: pre-processed okara; 1p corresponds to one passage through the homogeneizer; all samples subjected to HPH were previously pre-processed).

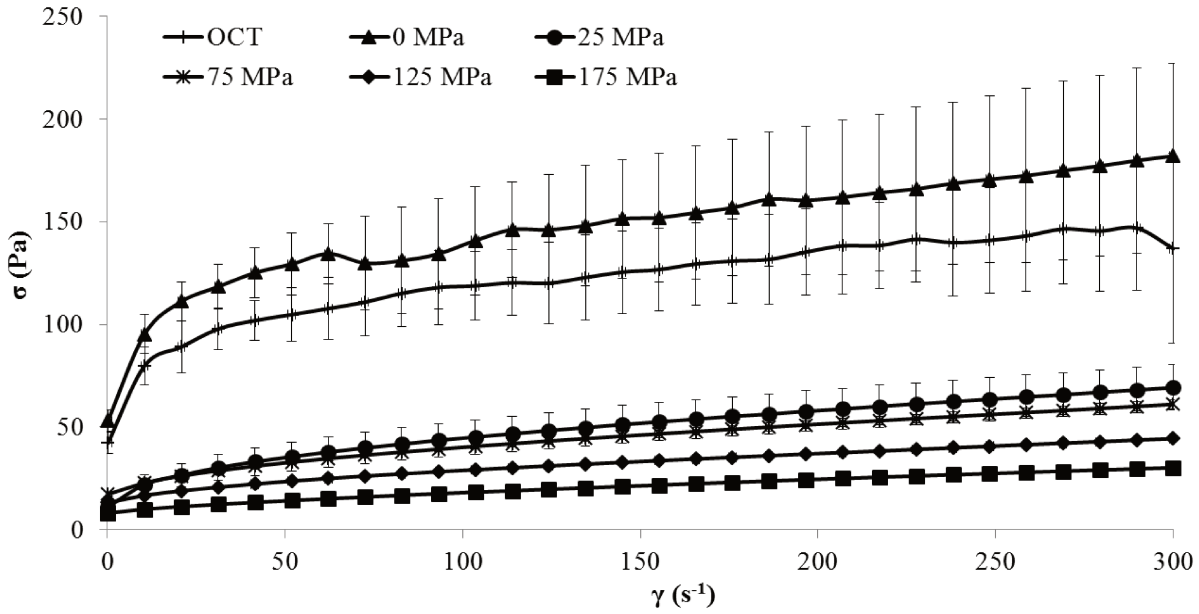

Fig. 3.3 Flow curves of okara subjected to high-pressure homogenization, under different pressure conditions (0, 25, 75, 125, or 175 MPa). Points represent mean values, and vertical bars represent standard deviations. (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed).

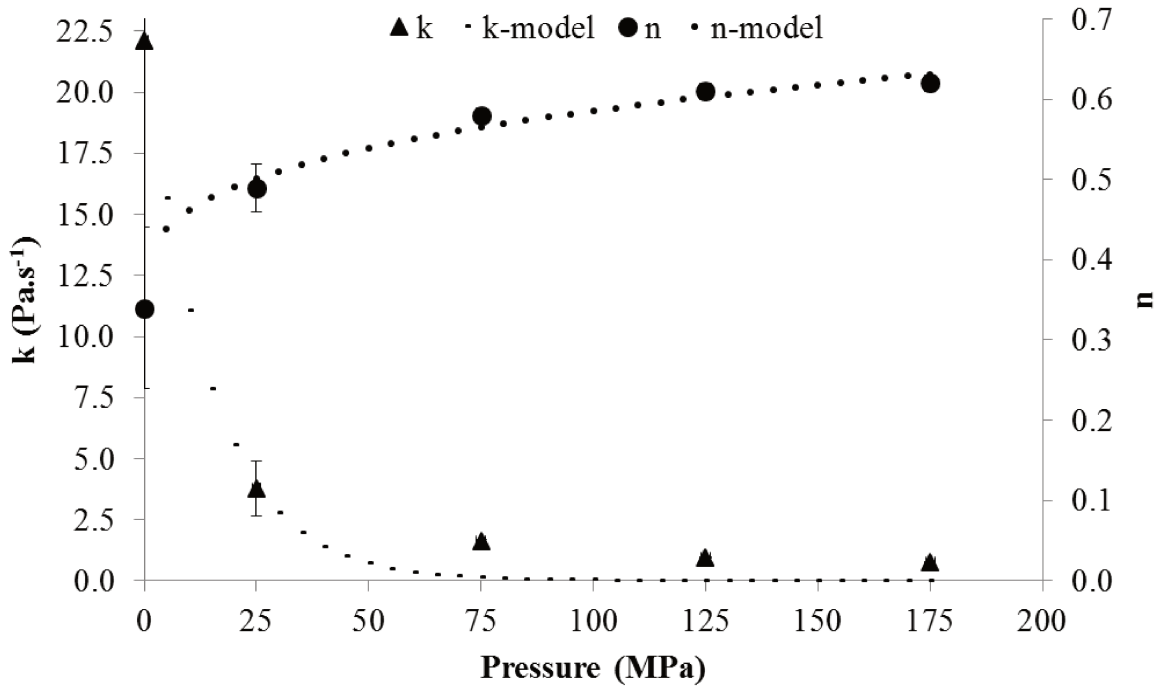

Fig. 3.4 Consistency index (k) and flow behavior index (n) of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa). Points represent mean values, and vertical bars represent standard deviations. (OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed).

The effect of the use of multiple passages on the flow behavior and the rheological parameters is shown in Fig. 3.5 and 3.6. Changes in rheology were observed as a result of the number of passages at 25 and 75 MPa, and to a lesser extent at 125 MPa, with no effect at 175 MPa. A reduction in $\sigma 0$ was observed only at 75 MPa after 2 passages (65%) ($p < 0.05$). Increasing the number of passages from 1 to 2 led to an increase in the parameter n (13-21%) ($p<0.05$) for pressures up to 25 MPa, while the increase from 2 to 3 passages led to a small but significant ($p < 0.05$) increase in n at 25 MPa-1p (~ 15%).

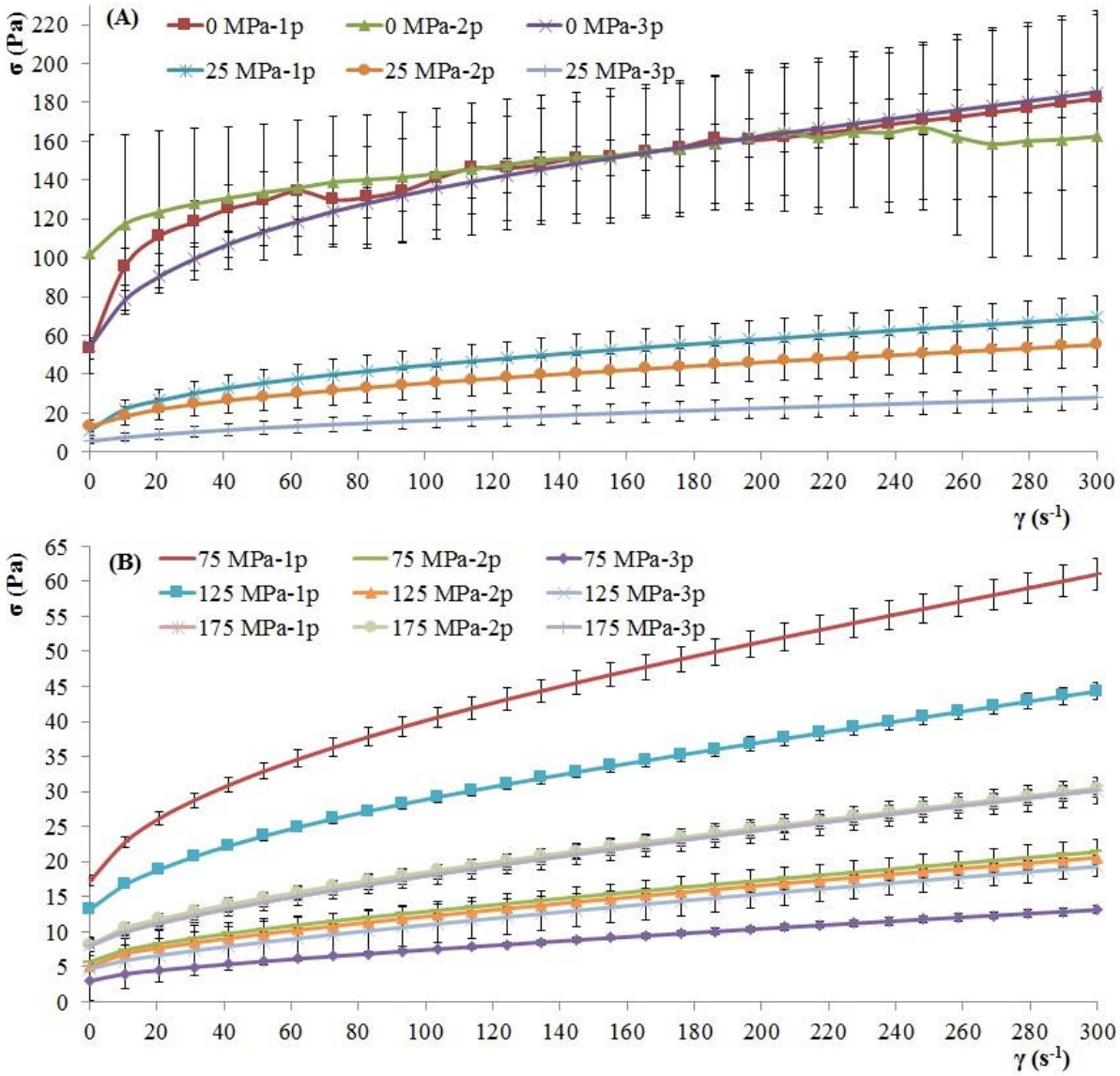

Fig. 3.5 Flow curves of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, 2, or 3). Points represent the mean values and bars the standard deviations. (all samples subjected to HPH were previously pre-processed; 1p, 2p, and 3p correspond to the number of passages).

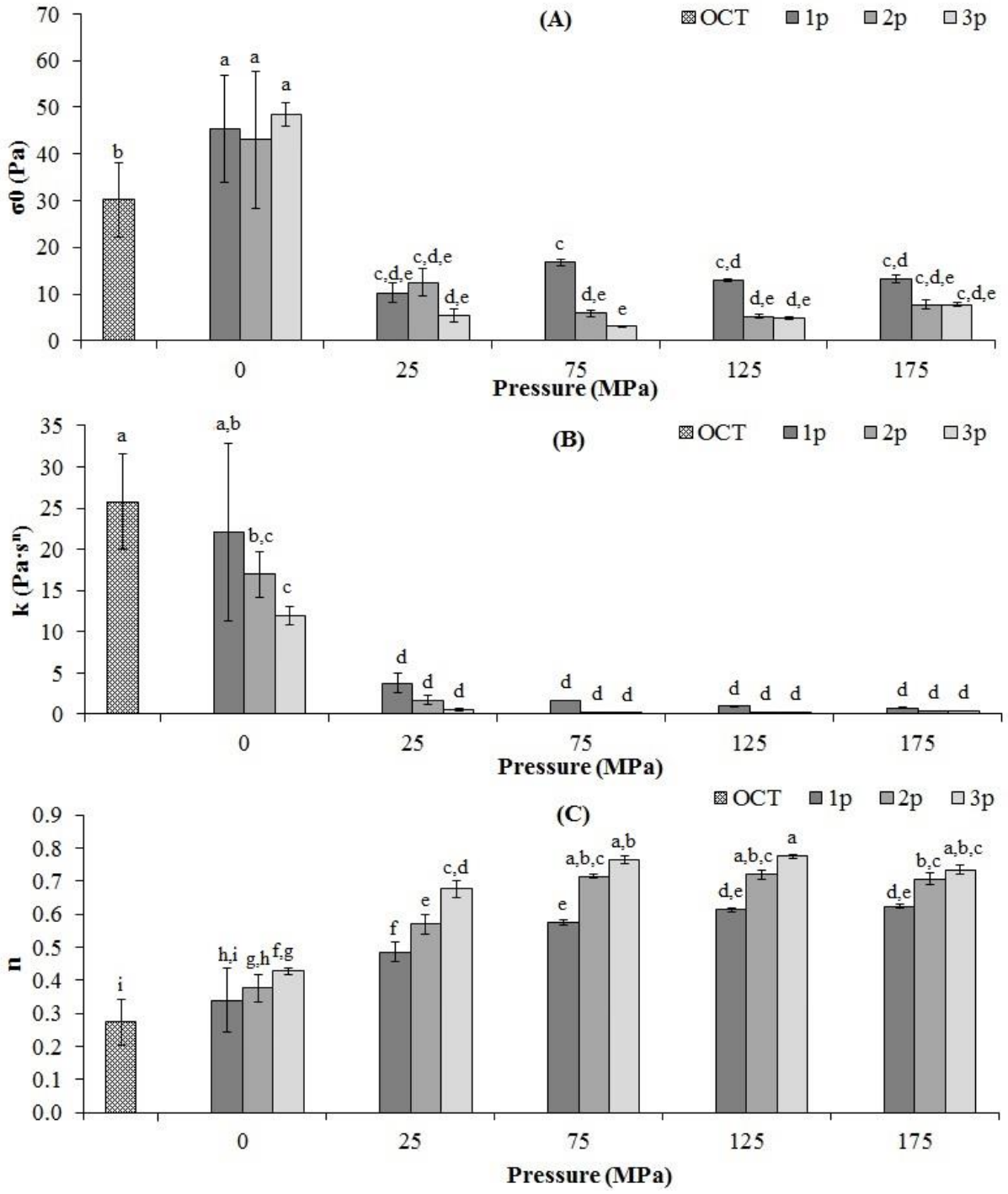

Fig. 3.6 Herschel Bulkley parameters values at 25 °C: (A) yield stress (σ0); (B) consistency index (k); (C) flow behavior index (n) of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, 2, or 3). Columns represent the mean values and bars the standard deviations. Means with equal letters do not differ statistically by the Tukey's test ($p > 0.05$). (OCT: pre-processed okara;

all samples subjected to HPH were previously pre-processed; 1p, 2p, and 3p correspond to the number of passages).

The application of single-pass HPH using pressures from 0 to 175 MPa led to a considerable reduction of the parameters k and σ0, and an increase in n, demonstrating that the samples were closer to the Newtonian behavior. The more significant changes were observed for σ0 and k using a single passage at 25 MPa, and n at 75 MPa. The use of multiple-passages (2p or 3p) led to changes in the rheological parameters of okara, which was more evident at the lower pressures studied (25 and 75 MPa).

The results of the rheological analysis suggest that HPH technology can provide better texture for okara, while the flow properties give information about the structure of the food during the industrial processing. Therefore, the present results can contribute to industrial planning and project design, providing information about operating instructions, including mixing, pumping, dosing, dispersion, and spraying, besides predicting the stability of okara-based products (Tomberg, 2017).

3.3.4 Effects of HPH on the visual appearance, color, and microstructure of okara

The application of HPH led to changes in the appearance of okara, as shown in Fig. 3.7. OST showed particles that separated easily from the aqueous phase while OCT and 0 MPa-1p were less heterogeneous, showing smaller particles but still easily separable from the aqueous phase. A more homogeneous consistency was observed for 25 MPa-1p and 25 MPa-2p with less perceptible and separable particles from the aqueous fraction. The sample 75 MPa-1p presented a homogeneous consistency, with no perceptible phase separation. From 75 MPa-1p, no differences were observed in the visual characteristics of okara.

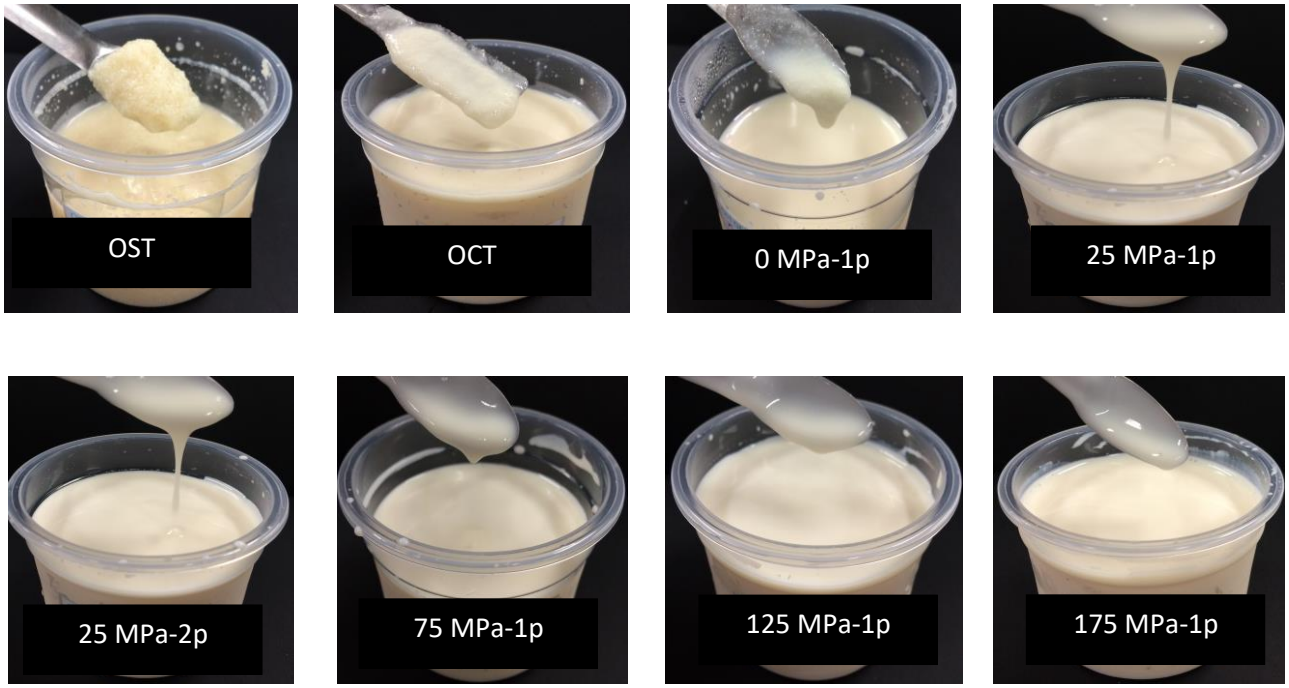

Fig. 3.7 Images of okara samples subjected to high-pressure homogenization under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, or 2). (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed; 1p and 2p correspond to the number of passages).

The color parameters, microstructures, extractable protein and fiber profile of the dispersions that presented the largest discrepancies among them in relation to particle size and consistency index were evaluated. The effect of HPH on the instrumental color parameters of okara was evaluated (Table 3.3). When comparing OST and OCT, an increase in a*, ΔE* (total color difference), and WI (whiteness index), and a reduction in b* were observed. After HPH processing, ΔE* reached values higher than 8.0, which corresponded to an intense perception of color changes. ΔE* indicates whether the color changes will be noticeable: no perception from 0 to 0.5, slightly noticeable from 0.5 to 1.5, noticeable perception from 1.5 to 3.0, very visible from 3.0 to 6.0, and intense perception for values above 6.0 (Cserhalmi, Sass-Kiss, Tóth-Markus, & Lechner, 2006). The increase in WI ($p < 0.05$) indicated that okara became whiter

with the HPH treatment due to the increase in the number of small particles, that leads to an increase in light reflection (Bernat et al., 2015).

Table 3.3 Instrumental color parameters a* b* L* (CieLAB system), total color difference (ΔE*), and whiteness index (WI) of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, or 2).

Treatment	L*	a*	b*	ΔE*	WI
OST	58.85 ± 1.37 (A)	-0.39 ± 0.09 (A)	9.42 ± 0.39 (A)	-	-
OCT	59.67 ± 1.93 (A) (c)	-0.05 ± 0.02 (B) (a)	8.51 ± 0.38 (B) (a)	2.46 ± 0.62 (c)	58.16 ± 2.44 (c)
0 MPa-1p	ND	ND	ND	ND	ND
25 MPa-1p	65.84 ± 3.19 (b)	-0.10 ± 0.02 (b)	7.30 ± 0.17 (b)	8.10 ± 1.95 (b)	65.06 ± 3.11 (b)
25 MPa-2p	66.80 ± 2.18 (a,b)	-0.09 ± 0.01 (b)	7.45 ± 0.26 (b)	8.43 ±1.38 (b)	65.76 ± 2.12 (b)
75 MPa-1p	67.93 ± 2.39 (a,b)	-0.09 ± 0.02 (b)	7.49 ± 0.58 (b)	12.25 ±1.84 (a,b)	67.06 ± 1.83 (a,b)
125 MPa-1p	70.42 ± 1.27 (a)	-0.08 ± 0.02 (a,b)	7.02 ± 0.24 (b,c)	12.25 ± 0.87 (a)	69.91 ± 0.84 (a)
175 MPa-1p	69.56 ± 1.55 (a,b)	-0.08 ± 0.02 (a,b)	6.63 ± 0.34 (c)	10.68 ± 1.53 (a,b)	68.29 ± 1.50 (a,b)

Results are mean of triplicates ± standard deviation. Means with equal upper case letters in columns do not differentiate between them by the t test ($p < 0.05$). Means with same uppercase letters in columns of same color did not differentiate between them by the t-test ($p > 0.05$). The values of OST and OCT were compared by t- test and equal uppercase letters show no difference between them. Means of all samples, except for OST, were compared using ANOVA and Tukey's test ($p > 0.05$); means with equal lowercase letters in columns of same color do not differentiate between them. Vertical lines represent the standard deviations. ND: not determined. (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed; 1p, and 2p correspond to the number of passages).

The soybeans microstructure is mainly composed of oval-shaped cotyledons cells consisting of lipids and protein bodies (Campbell et al., 2011; Rosenthal, Pyle, & Niranjan, 1998). Protein bodies occupy most of the cell volume and contain 80% protein, once the lipid bodies are much smaller than the protein bodies and fill the space between them (Rosenthal et al., 1998). These cell structures and their components were still observed in okara microstructure. As shown in Fig. 3.8, the intact cell structures, clearly observed in OST, were less frequent in OCT, which reveals that the pre-treatment modified the microstructure. At 25 MPa-1p, intact cell structures were rarely observed and were absent after processing at higher pressures. The sample 25 MPa-2p showed characteristics similar to 75 MPa-1p and 125 MPa-1p, while the sample 175 MPa-1p exhibited a different structure, with a less dispersed proteinaceous material, forming a continuous structure.

These findings are in accordance with the PSD (Fig. 3.1 and 3.2), which showed the effect of HPH on the reduction of particle size of okara. During the HPH, the okara structures are forced through a narrow gap and thus subjected to shear, friction, turbulence, cavitation, and intermolecular impact phenomena, followed by an abrupt decompression (Yan, Kun, Yang, Li, & Xiangeng, 2015). The homogenization led to a rupture of the remaining cells and breaking of cell fragments. Small cell fragments are less susceptible to break than larger ones or whole cells (Leite, Augusto, & Cristianini, 2014). This limited breaking is independent of the initial particle size, and may be due to the physical limitation of the homogenizer, once the gap dimensions and the resulting shear stress are inherent to the equipment, and higher tensile stress is required for the breakdown of okara cells/fragments when compared to the stress provided by the equipment.

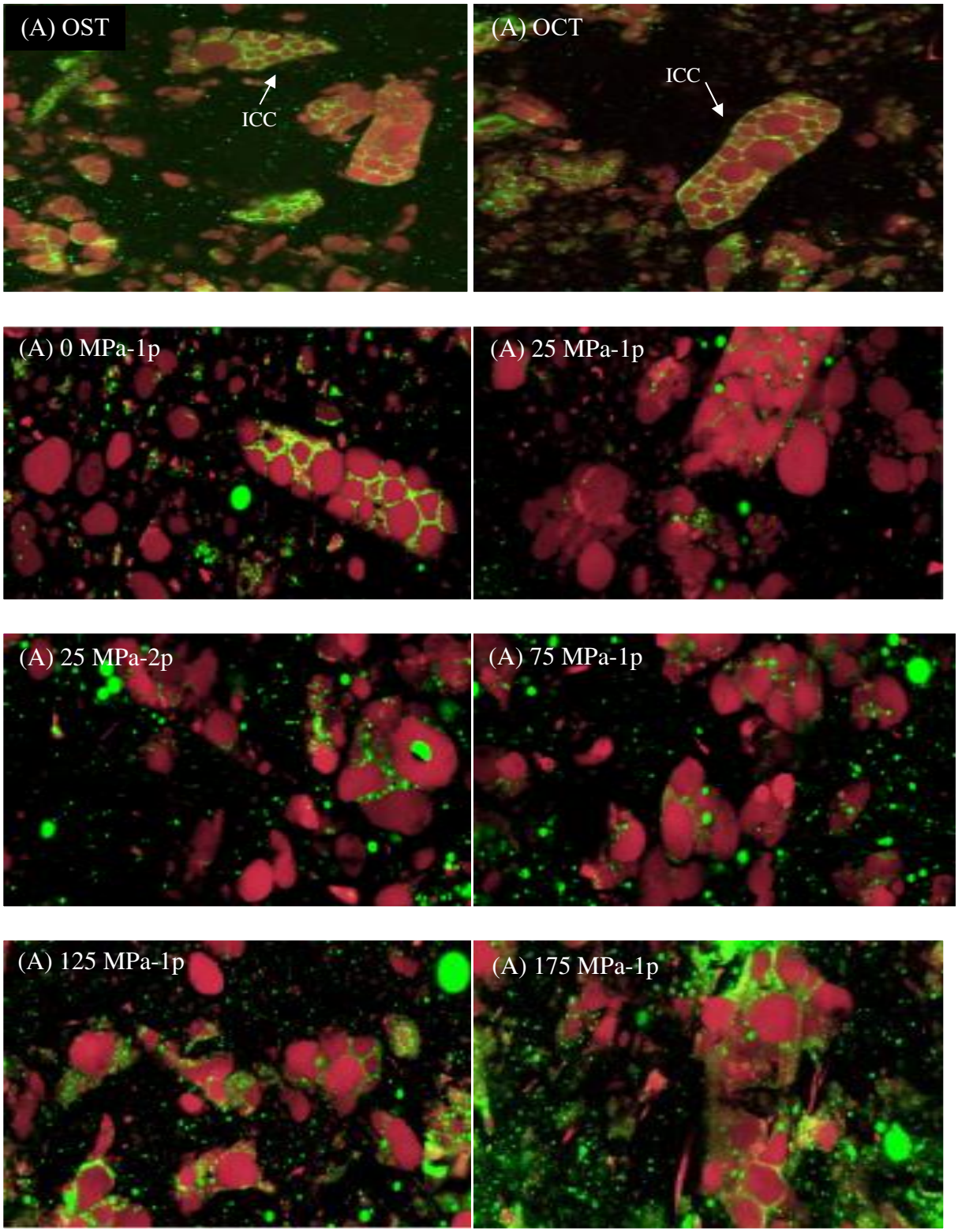

Fig. 3.8 Micrographs of okara subjected to high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, or 2). Images obtained with confocal microscopy. Visualization with fast green and red Nile, with

protein stained red, and lipids stained green. Intact cotyledon cell (ICC) is shown in the image. (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed; 1p, and 2p correspond to the number of passages).

3.3.5 Effects of HPH on extractable protein and fiber profile

The amount of extractable protein increased after the pre-treatment and HPH processing, as a function of the homogenization pressure ($p < 0.05$) (Fig. 3.9). OST showed low extractability, which was expected since it is a residue from the extraction of soluble proteins (Vishwanathan, Singh, & Subramanian, 2011). The maximum extractability of okara proteins was obtained at 175 MPa, which was 89% higher than that observed for OCT. The increase from one to two passages at 25 MPa led to a 21% increase in extractability indice. These findings may be due to the breakdown of cotyledon cells and their fragments and the release of proteins previously trapped in cellular structures, which still remained in the untreated okara (Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010). Interaction of proteins with other components such as fibers (Sasaki, Fang, Fukushima, Adschiri, & Arai, 2000) and lipids (Surel & Couplet, 2005) and also denatured protein during WSSE production (Chan & Ma, 1999) may have been dissociated by the HPH. The present results are in accordance with Porfiri, Vaccaro, Stortz, Navarro, Wagner, & Cabezas (2017), who used HPH as a pre treatment to solubilize the components of okara.

One of the reported effects of high pressure treatment is the modification of the fiber profile (Huang, He, Zou, & Liu, 2015; Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010; Ulbrich & Flöter, 2014), which may affect the functionality of the processed product (Tejada-Ortigoza, Garcia-Amezquita, Serna-Saldívar, & Welti-Chanes, 2015). Therefore, to evaluate whether the differences among the samples would be related to their fiber profile, we evaluated soluble and insoluble fiber contents of the samples 25-1p, 25-

2p, 125-1p, and 175 MPa-1p. No changes were observed for the fiber profile with increasing homogenization pressure and the number of passages at 25 MPa (results not shown).

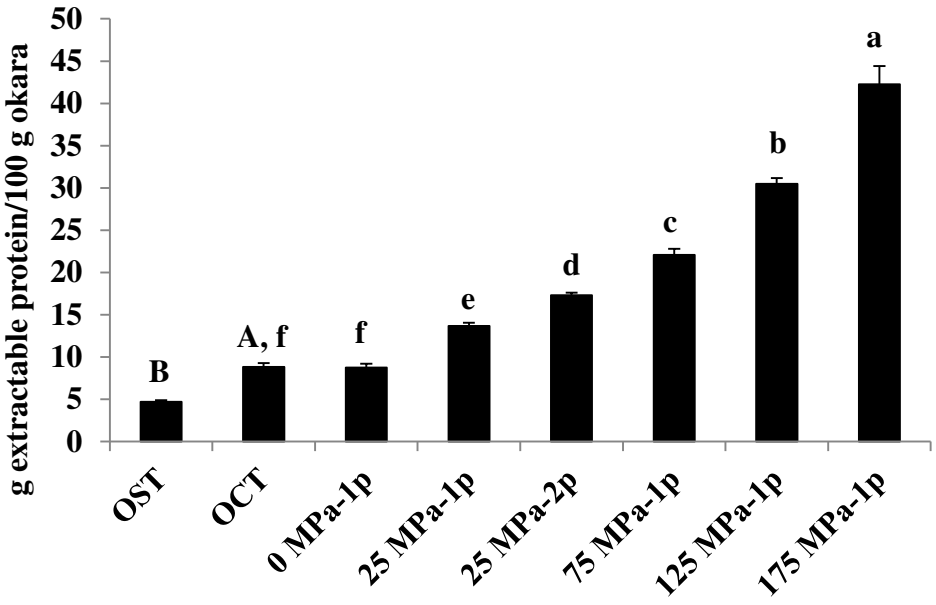

Fig. 3.9 Effect of high-pressure homogenization (HPH) under different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1, or 2) on extractable proteins of okara. Results are mean of triplicates ± standard deviation. The results of OST and OCT were compared by t- test and equal uppercase letters show no difference between them. Means of all samples, except for OST, were compared using ANOVA and Tukey's test ($p > 0.05$); means with equal lowercase letters in columns of same color do not differentiate between them. Vertical lines represent the standard deviations. (OST: untreated-okara, OCT: pre-processed okara; all samples subjected to HPH were previously pre-processed; 1p, and 2p correspond to the number of passages).

Possibly, the changes in σ0 and the other rheological parameters were due to the increase in the number of small particles (Fig. 3.1 and 3.2), wich can fill the space between the remained larger particles, leading to a "lubricant" effect, which decreases the resistance to flow

and changes the consistency index (Rojas, Leite, Cristianini, Alvim, Augusto, 2016; Servais, Jones, & Roberts, 2002). In addition, small particles require less energy to flow, which may explain the flow behavior of okara after the HPH processing. The increase in protein concentration in the aqueous fraction and its concomitant decrease in the insoluble matrix may also have contributed to changes in rheological parameters.

3.4. Conclusion

The HPH caused cell disruption and breaking of cell fragments into small particles, which led to a particle reduction, changed the particle size distribution and increased protein extractability, which may explain the changes observed in the rheological properties of okara. The reduction in the consistency index occurred from at the lowest pressure studied (25 MPa), which can be an advantage from the economic point of view. The results showed that the use of HPH generated a homogeneous material that can be used as an ingredient in food formulation, which can improve the nutritional value by adding fiber and high-quality proteins, and other compounds with a broad range of bioactivities, including phenolic compounds. The reduction of okara consistency observed in this study can lead to improvements in okara texture, thus allowing its use as a food ingredient. The rheological parameters can help in the process design and optimization, improving the handling and process operations, such as pumping, mixing and heat exchanging, among others.

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Conflict of interest

The authors declare that there is no conflict of interest.

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CAPÍTULO 4: ARTIGO 2 – EFFECT OF HIGH-PRESSURE HOMOGENIZATION ON THE BIOACTIVE POTENTIAL AND NUTRITIONAL PROPERTIES OF AN AQUEOUS DISPERSION OF OKARA

Short title: Effect of high-pressure on bioactive and nutritional properties of okara

Artigo submetido ao Food Bioscience

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ABSTRACT

This study investigated the effect of high-pressure homogenization on the nutritional properties and bioactive potential of an aqueous dispersion of okara, once this technology is capable of altering the physical properties of this agro-waste. The content of reactive substances to the *Folin-Ciocalteu* reagent increased by up to 66%, the values of ferric reducing antioxidant power up to 28%, with no changes in the oxygen radical absorbance capacity assay. Homogenization led increase to 89% and 12% in protein solubility and digestibility, respectively. The microstructure and the molecula mass profile of peptides of the digests were altered. The changes in the microstructure observed in previous research, with the disruption of okara cells/fragments, may have led to the release and solubilization of compounds. Besides the improvement of physical characteristics of okara, there was an increase in bioactive and nutritional properties, making okara a valuable byproduct for the utilization in the food industry.

Keywords: high dynamic pressure, antioxidant activity, digestibility, soy residue.

4.1 Introduction

Okara is an insoluble, low-cost, and abundant soybean curd residue generated during the production of water-soluble soybean extract (WSSE) (Multiterno et al., 2017; Pérez-López et al., 2017; Li et al., 2012a), and contains 49% fiber, 33% protein, 20% lipids, 7% soluble carbohydrates, and 3% ash on a dry basis (d.b.) (Lopez et al., 2005). These components have proven to exert several physiological functions, such as antioxidant (Li et al., 2012a; Jiménez-Escrig et al., 2008), hypocholesterolemic, hypoglycemic, and antihypertensive activities (Villanueva et al., 2011, Jiménez-Escrig et al., 2008, O'Toole, 1999). Despite the high nutritional value and bioactive potential of okara, this by-product has been discarded or used

only in animal feed (Vong and Liu, 2016), with an unexplored use as a food ingredient (Surel and Couplet, 2005). Therefore, several studies have been carried out to stimulate its use in human food (Li et al., 2012), thus leading to a lower environmental impact, reduction of treatment costs, diversification of production (Li et al., 2012; Mateos-Aparicio et al, 2010) and utilization of nutrients and bioactive compounds from okara (Jankowiak, et al., 2014).

However, the heterogeneous particulate matter of okara impairs sensory acceptability, including the texture profile of the products. The texture attributes can be improved by choosing a suitable processing technology (Wei et al., 2018), which can also contribute to preserving or enhancing the functional and nutritional quality of okara (Surel and Couplet, 2005). These technologies can modify the characteristics of the food matrix, and affect the digestion of nutrients (Nyemb-Diop et al., 2016; Turgeon and Rioux, 2011), facilitating or limiting nutrient release (Mat et al., 2015).

The structure of plant foods plays an important role on digestion process (Bhattarai et al., 2018), as the cell wall may limit the bioaccessibility of nutrients (Singh and Gallier, 2014). In the case of proteins, when encapsulated within cell walls, they are not hydrolyzed by the digestive enzymes of humans (Bhattarai et al., 2018). In addition, other factors such as viscosity and fiber content can modulate the kinetics of protein digestion and the release of peptides and amino acids in plasma (Fafaungwithayakul et al., 2011; Turgeon and Rioux, 2011). The protein release during digestion depends on several factors, including the protein structure, the interactions within the food matrix, and the process occurring in the gut (Singh and Gallier, 2014). Therefore, the product matrix should be considered when studying the nutritional value of food, aimed at developing new formulations and establishing processing conditions (Turgeon and Rioux, 2011; Parada and Aguilera, 2007).

High pressure is food preservation and transformation technology that maintains or enhances the sensory, nutritional (Mor-Mur, 2010) and functional (Yordanov and Angelova,

2010) characteristics of the processed product. It can improve the technological properties of leguminous plants, modify protein digestibility (Estrada-Girón et al., 2005) and provide new texture characteristics to the products (Torrezan and Cristianini, 2005). High-pressure homogenization (HPH) is a technology with the potential to be used in the manufacture of okara. In an earlier study, our research group investigated an aqueous dispersion of okara containing 5% protein (w/v) subjected to HPH and observed the okara cell rupture and breakage of fragments, which led to a particle size reduction and changed the particle size distribution. The lumpy appearance of okara, with particles visibly separated from the aqueous phase, became homogeneous and fluid. There was a change in the rheological parameters of okara, with a reduction of the consistency index, thus allowing obtaining a homogeneous material, with better incorporation in food systems (unpublished results).

Thus, the objective of the present study was to investigate the effect of HPH on the nutritional characteristics and the bioactive potential of an aqueous dispersion of okara. The relationship between the microstructure of the dispersion caused by HPH and the *in vitro* gastrointestinal digestion of proteins was evaluated.

4.2 Material and methods

4.2.1 Material

Soybean (*Glycine max L. Merril*) variety IAC Foscarin 31 was purchased from the Agronomic Institute of Campinas (Campinas, SP, Brazil). The enzymes pepsin and pancreatin used in gastric digestion, Folin-Ciocalteu reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri 2-pyridyl) -1,3,5-triazine (TPTZ), 2,2-azobis-2-methylpropionamidine dihydrochloride (AAPH), sodium fluorescein, tricine,

sodium dodecyl sulfate (SDS), and Nile red were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA), acetonitrile, β-mercaptoethanol, Coomassie G250 brilliant blue dye, sodium hydroxide, urea, and sodium azide were purchased from Merck (Hohenbrunn, Germany). Bis-acrylamide was purchased from Amresco (Solon, Ohio, USA). Acrylamide and Tris base were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals and reagents were of analytical or chromatographic grade.

4.2.2 Production and characterization of okara

The WSSE processing to obtain okara was performed in the laboratory, according to the procedures used in the WSSE processing industry (Benedetti and Falcão, 2003). Briefly, processing consisted of the following steps: grain washing in running water, immersion in water (1:7 w/v) for 30 minutes at room temperature, draining, peeling, immersion in a hot water bath (1:7 w/v; 97ºC) for 14 minutes, draining, milling in an industrial blender for 5 minutes using water at 60ºC (1:7 w/v), and filtration through a synthetic fabric to obtain okara, which was vacuum-packed and stored at -18 °C until use. Soybeans and okara were characterized for moisture, protein, ash (AOAC, 2000), lipids (AOAC, 2006), and total carbohydrates (calculated by difference). The amino acid composition of okara was determined by RP-HPLC after derivatization with phenyl isocyanate, according to the methodology described by White et al. (1986).

4.2.3 High-pressure homogenization (HPH)

After thawing, okara was diluted in water to reach a protein content of 5% (w/v), which was established as the maximum adequate concentration to be processed in the homogenizer. An aqueous dispersion consisting of 40 g okara and 60 g water was made, which was pre-processed in a high shear mixer (Ultra Turrax T-18 Basic, IKA Labortecnik,

Wilmington, NC) at 24,000 rpm/min for 5 minutes. The pre-processing dispersion was subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) using a Panda Plus 2000 homogenizer (GEA NiroSoavi, Italy) at a flow rate of 9 L/h, using a single passage for all pressure conditions, except for the treatment at 25 MPa, which was also subjected to two passages. The samples were identified as abbreviations referring to the pressure and number of passages, as follows: 0 MPa-1p, 25 MPa-1p, 25 MPa-2p, 75 MPa-1p, 125 MPa-1p, and 175 MPa-1p. The choice of these pressure conditions and the number of passages was based on an earlier study, which demonstrated that these conditions provided okara with different particle size and rheological properties (unpublished results).

The homogenizer was fed with the samples at 20 ± 5°C, and the processed material was immediately cooled in an ice bath for 15s. Okara subjected to 25 MPa-2p was cooled before the next pass through the homogenizer, once the pressure rise led to an increase in okara temperature, thus the samples processed at 0, 25, 75, 125, and 175 MPa reached the final temperatures of 19 ± 3°C; 20 ± 3°C; 24 ± 3°C; 26 ± 3°C and 37 ± 3°C, respectively.

The samples were stored at 7 ± 3°C immediately after processing and analyzed within 24 hours for solubility in water and gastrointestinal digestion *in vitro*. Part of the processed samples and the aqueous extracts from the protein solubility analysis were kept at -18°C (maximum 7 days) to be used in the determination of antioxidant activity and electrophoresis profile. The experiment was performed in three replicates, and two controls were made using pre-processed okara (OCT) or untreated okara (OST).

4.2.4 Antioxidant activity

The aqueous extracts obtained in triplicate in the determination of protein solubility in water (Section 2.5) were used to determine the antioxidant activity after adjusting the protein concentration to 15 mg/mL. The determination of the *Folin-Ciocalteau* reagent reducing

substances (FCRRS), and the Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorption Capacity (ORAC) assays were performed in triplicate in a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Vermont, USA).

The FCRRS assay was performed according to the methodology described by Medina (2011). An aliquot of both the sample extract and water (totaling 500 µL) and 50 µL of *Folin-Ciocalteau* reagent was placed in a microtube. After stirring and standing for 5 minutes, 500 µL of 7% (w/v) sodium carbonate and 200 µL of water was added, followed by stirring and resting for 90 minutes. A standard curve was constructed with a gallic acid solution in water (50, 100, 200, 300, 400, 500, and 600 µg/mL) and negative control was done by replacing the sample extract or standard with water. After the reaction time, three aliquots of 200 µL were transferred to a microplate, followed by immediate absorbance readings at 765 nm and 25°C. The results were expressed as µg of gallic acid equivalent (GAE)/g sample.

The FRAP assay was performed according to the methodology described by Rufino et al. (2006). An aliquot of both the sample extract and water (totaling 120 µL) and 900 µL of FRAP reagent (450 µL 0.3 M acetate buffer, pH 3.6, 225 µL of 10 mM TPTZ, and 225 µL of 20 mM $FeCl_3$) was placed in a microtube. After stirring, three aliquots of 200 µL were transferred to a microplate. The standard curve was constructed with Trolox solution in ethyl alcohol (25, 50, 100, 200, 300, and 400 µg/mL) and negative control was done by replacing the sample extract with water. After 30 minutes, the absorbance readings were made at 595 nm and 37°C, and the results were expressed as µmol Trolox equivalent (TE)/g sample.

The ORAC assay was performed according to the methodology described by Dávalos et al., (2004). For that, 20 µL of sample extract, 120 µL of fluorescein (diluted in potassium phosphate buffer pH 7.4 at the final concentration of 0.378 µg / mL, prepared from a stock solution of 3.87 mg / mL) and 60 µl of AAPH at 108 mg/mL in water were mixed. A standard curve was constructed with Trolox solution in potassium phosphate buffer (0.1, 1.0,

10, 25, 50, 80, and 100 μM) and the negative control was done by replacing the sample extract with potassium phosphate buffer. Fluorescence readings were performed in triplicate at 37°C, immediately after the addition of the reagents on the microplate, every 1 minute, for 80 minutes, using emission and excitation wavelengths of 520 nm and 485 nm, respectively. AUC (area under the curve) of the extract concentrations and the control was calculated by using the fluorescence readings, according to Eq. 1. The blank AUC was subtracted from AUC of each sample and standard. The AUC of the samples were then calculated by the equation obtained with the Trolox standard curve and the values were expressed in Trolox equivalents (μmol TE/g dry sample).

$$AUC = 1 + \frac{f_2}{f_1} + \frac{f_3}{f_1} + \frac{f_4}{f_1} + \frac{f_n}{f_1} \quad (1)$$

where f_1 is the fluorescence reading at time 1; f_2 is the fluorescence reading at time 2; f_3 is the fluorescence reading at time 3; f_4 is the fluorescence reading at time 4; f_n is the fluorescence reading at the time of 80 minutes.

4.2.5 Characterization of okara proteins

The water-soluble proteins of okara were determined according to Morr et al. (1985). Briefly, 15 g of okara was dispersed in 40 mL of distilled water and kept under magnetic stirring for 1 hour at room temperature. The dispersion was transferred to a 50 mL volumetric flask, the volume was completed with distilled water and centrifuged (20,000xg for 30 min at 4°C). The total nitrogen content was determined in the aqueous extracts by micro-Kjeldhal (AOAC, 2000) to determine the protein concentration (mg/mL). Protein solubility was calculated according to Eq. 2

$$\text{Protein solubility in water (\%)} = \frac{(A \times 50)}{W \times \left(\frac{S}{100}\right)} \times 100 \quad (2)$$

where A is the protein concentration of the supernatant (mg/mL); W is the sample mass (mg) and S is the protein concentration of the sample (%).

The electrophoretic profile of the aqueous dispersion of okara was determined in SDS-PAGE under reducing conditions (Laemmli, 1970) using Mini-Protean II (Bio-Rad, Hercules, CA, USA) and separating and stacking gels with 12% and 4%. polyacrylamide, respectively. Samples were diluted (0.4% w/v protein) in reducing buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.1% bromophenol blue, pH 6.8) and heated to 90 °C/5 min. Aliquots of 20 μL sample were applied to each well. After the run, the gels were stained in Coomassie Blue G250 (0.1% w/v) and decolorized with acetic acid /methanol/water (1:4:5). A standard molecular weight marker kit for 16 to 94 kDa (Bio-Rad, Hercules, CA, USA) was used to estimate the MM of the samples.

The digestion *in vitro* was performed in two stages to simulate the gastric digestion (GD) and intestinal digestion (ID), as reported by Minekus et al. (2014), with adaptations. The activity of the enzymes pepsin (Sigma P6887) and trypsin in pancreatin (Sigma P1625) was determined according to the protocol for enzymatic assays (Minekus et al., 2014), and the values were 4,964 U/g and 1.79 ± 0.28 U/mg, respectively. For the digestion assay, an enzyme:protein ratio of 1:20 (w:w) was used for pepsin and pancreatin. To evaluate the effect of the reagents, enzymes, and samples on the digestion assays, two controls were made as follows: a reagent control (RC) containing gastric and intestinal fluids and gastrointestinal enzymes, without the addition of sample, and a sample control (SC) containing fluids and sample, without the addition of enzymes. After 120 minutes (final of GD) and 240 minutes (final of GD + ID), aliquots were taken to determine the protein digestibility. The aliquots obtained after the 240 minutes of the digestion process were also evaluated for the molecular

mass profile (MM) of the peptides by size exclusion high-performance liquid chromatography (SEC-HPLC) and microscopic evaluation.

Protein digestibility was determined after GD, and ID, based on the nitrogen content after precipitation with 13% (w/v) TCA (Chen et al., 2015). For that, 2 mL of 20% TCA (w/v) was mixed with 1 mL of digest and, after 10 minutes of rest, the samples were centrifuged at 5000xg for 5 min at 25 °C (Centrifuge RC5C, Sorvall Instruments Dupont, Wilmington, USA). Nitrogen was determined in the supernatant fraction (AOAC, 2000) and the digestibility was calculated considering the contents of total nitrogen and soluble nitrogen, according to Eq. 3.

$$\text{digestibility } (\%) = \frac{N_D - N_{CA} - N_{CR}}{N_t - N_{CA}} \times 100\ \% \quad (3)$$

where N_D = nitrogen soluble of the digest (mg); N_{CA} = nitrogen of the control sample, without the addition of enzyme (mg); N_{CR} = nitrogen content of the control containing only the enzymes and (mg); and N_T = total nitrogen content of the sample (mg).

The urease activity index (UI) was determined as described by AOCS (2009) to investigate the presence of antinutritional factors.

The MM distribution of the digests was evaluated by SEC-HPLC. The analysis was performed using the Agilent HPLC system (Waldbronn, Germany), equipped with a quaternary, semi-preparative and analytical pump system, DOD Array Detector (DAD) and TSK-gel G 2000SWXL column (7.8 mm, 300 mm - TosohBioSep - Montgomeryville, PA, USA). The mobile phase was acetonitrile /Mili-Q water (25:75, v:v) with 0.1% TFA, using a 0.45 μm membrane. Aliquots of 10 μL were injected into the column and elution was performed at 25°C, at a flow rate of 0.4 mL/min, monitoring at 214 nm. The samples were diluted in Mili-Q water with 0.1% TFA, to the protein concentration of 4.5 mg/mL and filtered using a 22 μm cellulose acetate membrane. The following protein standards were used to estimate the MM of the

peptides: triosephosphate (26.625 kDa), myoglobin (16.950 kDa), α-lactalbumin (14.437 kDa), aprotinin (6,512 kDa), insulin (3,496 kDa) and bacitacine (1,423 kDa) (Bio-Rad, Hercules, CA, USA). The relative percentage of each range (6.5-14.4, 3.5-6.5, 1.4-3.5 and <1.4 kDa) was determined by the relation between the peak area and the corresponding MM and the total area. Star Chromatography Workstation (Agilent) software was used to record and process data.

4.2.6 Microstructure of okara and its digests

The microstructure of the samples was analyzed by confocal laser scanning microscopy, in Axiovert 200 M microscope with LSM 510 scanning module equipped with an argon laser (458, 477, 488, and 514 nm) and helium-neon (543 and 633 nm). Fast green (FGF) and Nile red (NR) were used to dye proteins and lipids, respectively, as described by Liu et al. (2019). The samples were prepared in microtubes as follows: 500 μL sample + 25 μL FGF (0.1% w/v) + 25 μL NR (0.234% w/v). The samples were agitated for 1 minute, left to rest for 5 minutes, and transferred to slides with a 10 mm depth cavity. The observations were performed using an oil-immersion objective (63x/1.4 NA/oil) and excitation wavelengths of 633 and 488 nm (He/Ne laser). All images were taken at a resolution of 1024 x 1024 pixels.

4.2.7 Statistical analysis

All analyses were performed in triplicate. Data normality was analyzed by the Kolmogorov-Smirnov test. The t-test, analysis of variance (ANOVA) and Tukey's test was used when necessary, at a significance level of $P < 0.05$ using the statistical package GraphPad Prism version 6.0 (GraphPad Software Inc., Califórnia, EUA).

4.3. Results and discussion

The moisture, protein, lipids, ash contents, and total carbohydrates (on a dry basis) were, respectively, 10.5 ± 0.2; 38.1 ± 1.1; 21.2 ± 0.8; 4.8 ± 0.0; and 35.9g/100g for soybeans and 73.9 ± 1.1; 47.9 ± 0.7; 21.8 ± 1.0; 2.7 ± 0.0; and 27.6 g/100 g for okara. The proximate composition and the amino acid composition of okara dispersion were similar to the values described by other authors (Kumar et al., 2016; Mext, 2015; Multiterno et al., 2017). As expected, okara presented high protein and low carbohydrate contents, once it was made from peeled soybeans. Similar to soybeans, okara also has an amino acid profile recognized as the most complete of all plant sources, with an amino acid pattern that resembles that of high-quality animal proteins, except for methionine and cysteine (Ma, 2004).

4.3.1 Effect of HPH on the antioxidant activity of okara

The total antioxidant activity of plant products cannot be evaluated by a single method due to the complex nature of the samples (Sbroggio et al., 2016) and the different mechanisms of action of antioxidants (Moure et al., 2001). Therefore, the antioxidant activity in this study was determined by three methods: FRAP, based on single electron transfer mechanism, and ORAC based on hydrogen atom transfer (Alves et al, 2010). The results of FRAP, ORAC, and FCRRS assays can be seen in Figure 4.1.

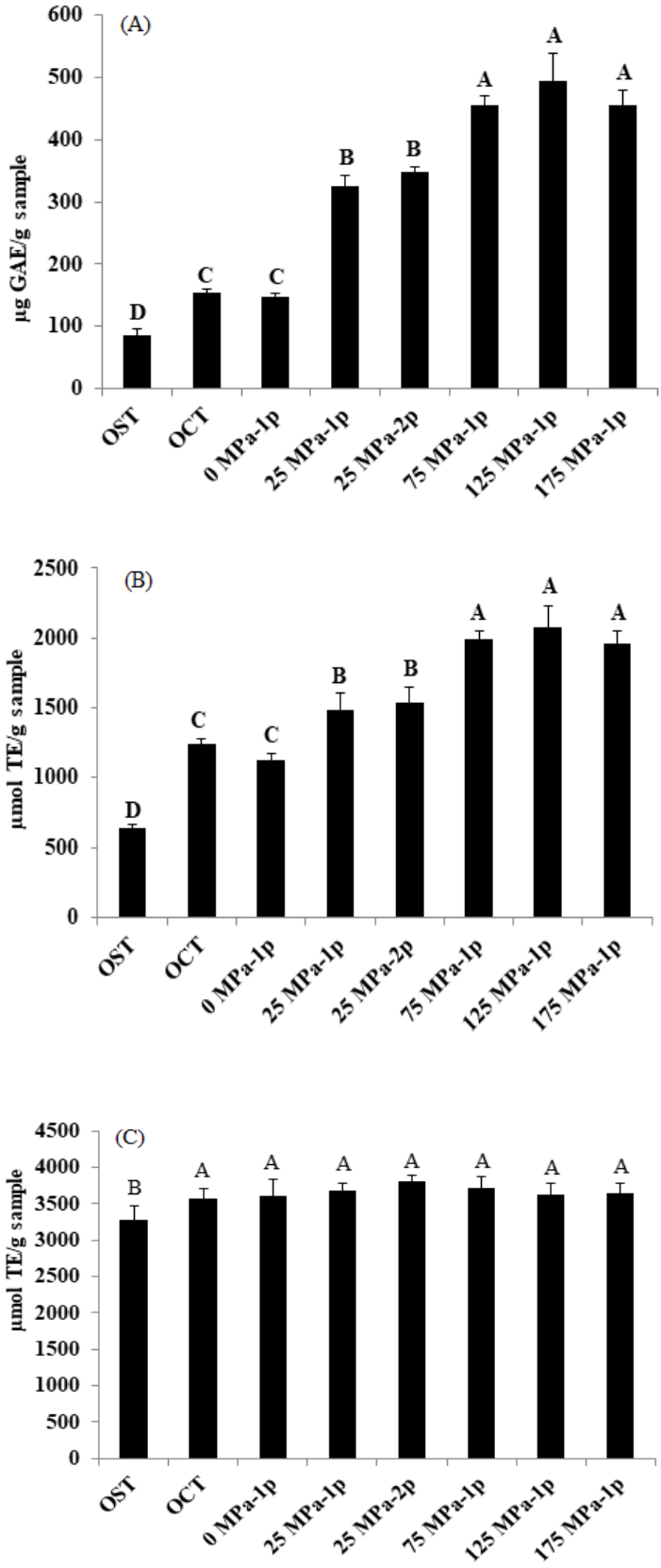


Figure 4.1. Folin Ciocalteau reagent reducing substances (FCRRS) (A) and the antioxidant activity determined by the FRAP (B) and ORAC (C) assays for the aqueous extracts of okara

subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1 or 2). Columns represent averages of values of three replicates, and bars represent the standard deviations. Columns with the same letters do not differ statistically by the Tukey's test ($p < 0.05$). GA: Gallic acid; TE: Trolox equivalent (OST: untreated okara, OCT: pre-processed okara, 1p and 2p corresponded to the number of passes).

An increase in FCRRS and higher antioxidant activities were observed by FRAP and ORAC assays, with values of 45, 49, and 8%, respectively, after the pre-treatment of okara, when compared to OST. The higher FCRRS and antioxidant activity determined by FRAP ($p < 0.05$) was not observed in the ORAC assay ($p > 0.05$). Regarding the OCT, the maximum increase in FCRRS (66%) and FRAP (38%) was found at 75 MPa, which was also observed at higher pressures. No changes were observed for FCRRS and FRAP values with the increase in the number of passages at 25 MPa ($p > 0.05$) (Figure 4.1).

These results demonstrate that the components responsible for the increase in the antioxidant activity acted predominantly by the electron donation mechanism. Silva et al. (2017) studied the antioxidant activity of flaxseed and reported that protein acts as antioxidants mainly by hydrogen transfer, while the phenolic compounds also act by the electron donation mechanism. Thus, probably the soluble phenolic fractions (present in cell vacuoles) and the insoluble fractions (found in cell walls or in association with other compounds) (Vong et al., 2017; Pandey and Rizvi, 2009) were released after the pre-treatment, especially with the use of HPH. The reduction of particle size of the pre-processed and HPH-treated okara (unpublished results) may have facilitated the release of components such as proteins and peptides (Wiboonsirikul et al., 2013), phenolic compounds (Rashad et al., 2011) and others, including α-tocopherol, γ-tocopherol, δ-tocopherol (Matsuo, 1997), coumestans, saponins, phytates (Li et al., 2012b), which may have led to the increase in FCRRS contents and/or antioxidant activity.

4.3.2 Effect of HPH on the properties of okara proteins

The electrophoretic profile determined by SDS-PAGE under reducing conditions of HPH-treated okara is shown in Figure 4.2. The protein profiles of okara were similar to those described for soybeans. The major protein fractions of soybeans, β-conglycinin 7S (a trimer with MM of 150 to 200 kDa) and glycine 11S (a hexameter with MM of 320 to 375 kDa) (Wang et al., 2008) were dissociated, with bands corresponding to α, α', and β subunits of β-conglycinin (about 58, 57, and 42 kDa, respectively) and acid and basic polypeptides of glycinin (37 to 45 kDa and 18 and 20 kDa, respectively) (Nakornpanom et al., 2010; Wang et al., 2008).

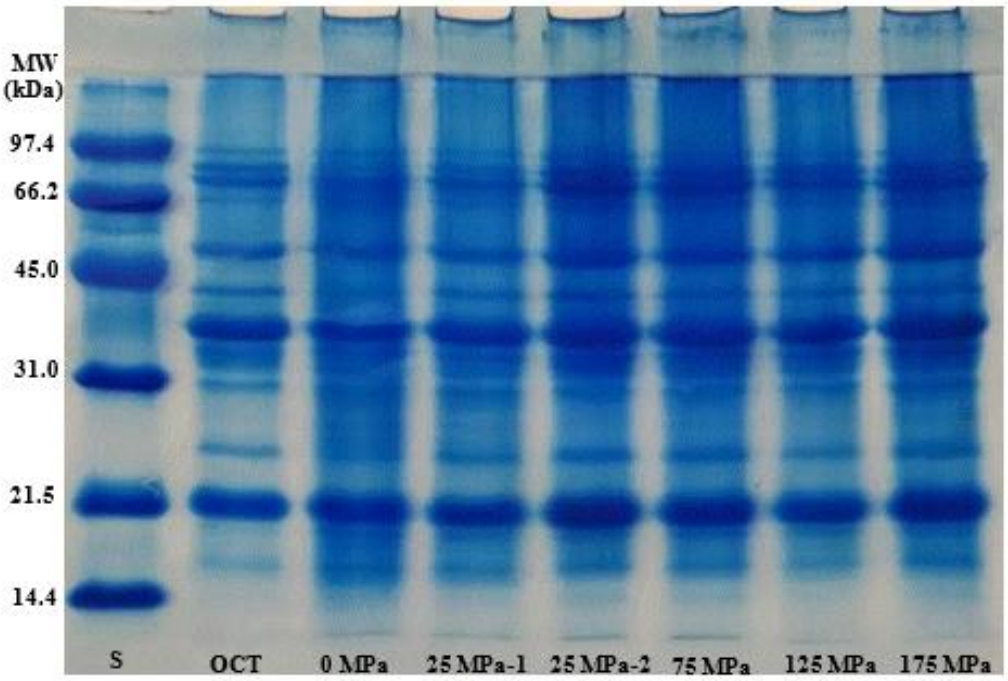


Figure 4.2. Electrophoretic profile by SDS-PAGE (12% polyacrylamide gel, under reducing conditions) of HPH-treated okara subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1 or 2). MM: molecular mass; P: MM standard (14.4 to 97.4 kDa). (OST: untreated okara; OCT: pre-processed okara).

Although no differences in the protein profile of HPH-treated okara were observed in the electrophoretic analysis, the solubility of protein in water increased in the pre-processed and HPH-treated sample, as a function of the homogenization pressure ($p < 0.05$) (Table 4.1). As expected, OST presented low protein solubility (4.7%), once okara is a byproduct resulting from the extraction of water-soluble components. The increase in protein solubility was probably due to the disruption of cotyledon cells and their fragments, leading to the exposure of protein fractions that were previously trapped within the cells. The solubility of protein from insoluble aggregates or the release of protein fractions may also have occurred. Other studies have also found that high pressure allows increasing the solubilization of components from soybeans and okara (Omi et al, 1996; Porfiri et al., 2017).

The results of protein digestibility of okara are presented in Table 4.2. The protein digestibility of the sample OST was 52.2%, which is similar to that reported by Kumar et al., (2016), who found a value of 48.5% when simulating digestion *in vitro* of dehydrated okara. No changes in protein digestibility were observed in the pre-processed okara in the final of both GD and GD + ID ($p > 0.05$). The protein digestibility of okara was also positively affected by HPH. The sample treated at 25 MPa exhibited 24% and 12% increase in digestibility at the end of GD and GD + ID, respectively, in relation to OCT ($p < 0.05$). Similar behavior was also observed for the samples treated at the highest pressures studied ($p > 0.05$). At 25 MPa, the increase from one to two passages in the homogenizer had no effect on protein digestibility ($p > 0.05$). Although the protein solubilization greatly increased with increasing pressure, up to 90%, a less significant increase was observed for the digestibility, with values up to 15%. Thus, it is suggested that the proteins were not totally accessible to the digestive enzymes, even when dissociated from the matrix.

The microscopic images revealed that, as a result of HPH, especially at 175 MPa, the protein fraction was covered by lipid droplets, whose diameter was reduced when compared

to the other samples. Therefore, this lipid coating, as well as a possible formation of protein aggregates, may have contributed to reduce the accessibility of digestive enzymes to proteins.

Tabela 4.1. Protein solubility in water of HPH-treated okara subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1 or 2) and protein digestibility after simulation of gastrointestinal digestion *in vitro*.

Treatment	Number of passages	% Protein solubility in water	% Digestibility GD	% Digestibility (GD+ID)
OST	-	4.7±0.2 ^{g}	21.3±2.0^{b}	52.2±1.4^{b}
OCT	-	8.8±0.5 ^{f}	23.7±1.6^{b}	51.5±1.1^{b}
0 MPa	1	8.7±0.5 ^{f}	23.8±1.1^{b}	54.1±2.0^{b}
25 MPa	1	13.7±0.4 ^{e}	31.2±2.1^{a}	58.7±1.1^{a}
25 MPa	2	17.3±0.3 ^{d}	28.7±1.6^{a}	59.6±2.1^{a}
75 MPa	1	22.1±0.7 ^{c}	30.2±1.5^{a}	58.7±4.1^{a}
125 MPa	1	30.5±0.6 ^{b}	30.0±1.6^{a}	58.6±4.0^{a}
175 MPa	1	42.2±2.28 ^{a}	28.7±1.7^{a}	58.5±1.7^{a}

Results presented as mean of three replicates ± standard deviation. Means with the same letters, in the same column, do not differ statistically from each other by the Tukey's test ($p > 0.05$). GD: gastric digestion; ID: intestinal digestion. (OST: untreated okara; OCT: pre-processed okara, 1p, and 2p corresponded to the number of passages).

The nutritional quality of soy products should be investigated due to the presence of antinutritional factors, and the determination of urease activity (UA) is an indirect parameter to assess these factors (Pinto and Castro, 2008). The UA of soybeans and okara were, respectively, 2.05 ± 0.04 and 0.02 ± 0.02, indicating that the heat treatment used in WSSE

processing was sufficient to reduce the antinutritional factors to adequate levels in okara (Wachiraphansakul and Devashastin, 2007; Wachiraphansakul and Devashastin, 2005). Therefore, the antinutritional factors, known to have the ability to inhibit protein digestion (Monteiro et al., 2004) possibly did not alter the protein digestibility of okara.

The MM distribution of okara digests obtained by HPLC is shown in Table 4.2. The digests OST and OCT presented peptides smaller than 1.4 kDa, as also observed for all the other digests, which exhibited peptides in this size range. In addition to the peptides lower than 1.4 kDa, the digest subjected to 0 MPa also had peptides between 1.4 and 3.5 kDa, while the digests treated at 25 MPa-1p, 25 MPa-2p, 75 MPa-1p, 125 MPa-1p, and 175 MPa-1p showed peptides between 6.5 and 14.4 kDa; the digest subjected to 175 MPa-1p also showed peptides between 3.5 and 14.4 kDa. These results suggest that, even with similar digestibility profile, the cleavage profile of the proteins by the digestive enzymes was altered by the pressure conditions, with a release of peptides of higher MM for the sample subjected to 175 MPa, which is related to the different accessibility of the enzymes to proteins due to the changes in microstructure promoted by HPH. The presence of peptides with different structural characteristics may lead to changes in their physiological functional properties.

Tabela 4.2. Molecular mass distribution* (MM) (%) of peptides after gastrointestinal digestion *in vitro* of HPH-treated okara subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1 or 2).

MM Distribution (kDa)	Area (%)							
	Control		Pressure (MPa) – Number of passages					
	OST	OCT	0	25-1p	25-2p	75	125	175
< 1.4	100	100	89.55	98.5	96.7	98.2	97.1	88.4
1.4 – 3.5	nd	nd	10.45	nd	nd	nd	nd	nd
3.5-6.5	nd	nd	nd	nd	nd	nd	nd	6.62
6.5-14.4	nd	nd	nd	1.5	3.3	1.8	2.9	5.0
14.4-26.6	nd	nd	nd	nd	nd	nd	nd	nd

nd = not detected. * MM distribution determined by SEC-HPLC.

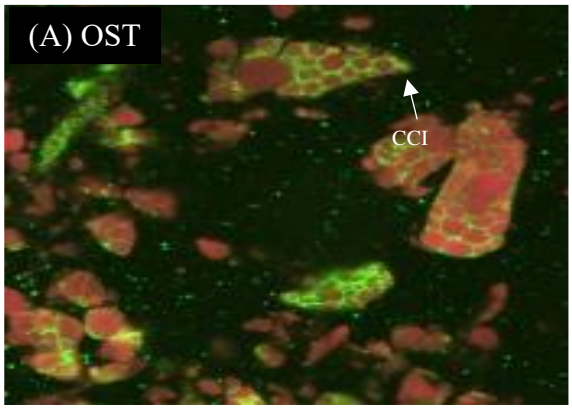
4.3.3 Microstructure of okara and okara digests

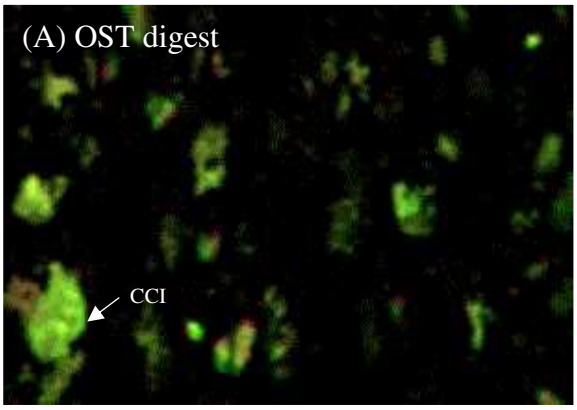
The impact of HPH on the microstructure of okara and its digests was evaluated by confocal microscopy. To assess the possible relationship between the changes in microstructure and protein digestibility, two dyes were used for staining proteins and lipids.

Figure 4.3 shows the microstructure of HPH-treated okara and the respective digests. The intact cotyledon cells frequently observed in okara (OST, OCT, and 0 MPa-1p) were less frequent or absent in the HPH-treated samples, and the fragments became smaller with increasing the homogenization pressure. The visualizations revealed a similar microstructure of okara samples subjected to up to 125 MPa. The sample treated at 175 MPa-1p presented microstructure characteristics different from the others, with less dispersed protein material, forming a continuous structure, covered with small lipid droplets. This difference suggests changes in the okara microstructure of this treatment, which may explain the difference

in the MM profile of the peptides of the digests (Table 4.2). The more continuous, less spread structure, covered by lipid droplets may have contributed to modify the enzymatic accessibility and, as a consequence, to have a profile of peptides different from the other samples. When comparing the micrographs of the HPH-treated samples and their respective digests, a similar microstructure profile was observed, with less protein material observed in the digests. The microscopic observations are in agreement with the protein digestibility results (Table 4.2), once the OST, OCT and 0 MPa samples, which frequently had intact cells, have less digested protein.

When the results are evaluated together, the cell disruption and the reduction of fragments due to HPH led to the exposure of proteins previously encapsulated within cell walls (Bhattarai et al., 2018; Singh and Gallier, 2014). HPH appears to have disrupted the interactions between proteins and other components such as fibers and other polysaccharides (Fafaungwithayakul et al., 2011; Turgeon and Rioux, 2011), which may have facilitated hydrolysis by the digestive enzymes, thus increasing their bioaccessibility. The results of this study suggest that the protein solubilization was not the crucial factor for the increase in protein digestibility, once a discrete increase in digestibility was observed even with higher homogenization pressures. Although the integrity of cell structures contributed to protein solubility, incomplete digestion of okara proteins was observed for all samples. Other authors have observed low protein digestibility in okara (Vong and Liu, 2016; Jiménez-Escrig et al., 2010), probably due to the presence of fibers. Bhattarai et al., (2017) subjected leguminous plants to simulated digestion conditions, and found that the addition of fibers from cell wall of leguminous plants reduced the hydrolysis of starch and proteins as a function of the amount of fiber, suggesting the existence of structural interactions between macromolecules that may affect the susceptibility to the enzyme activity.


(A) OST
CCI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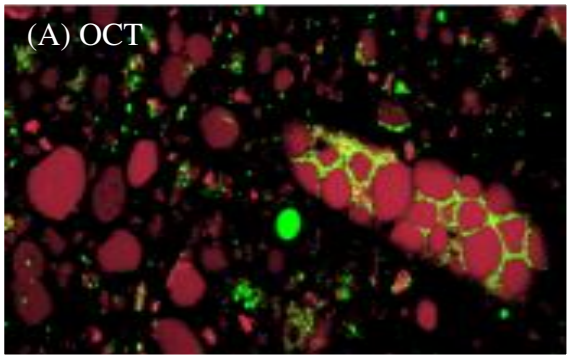
CCI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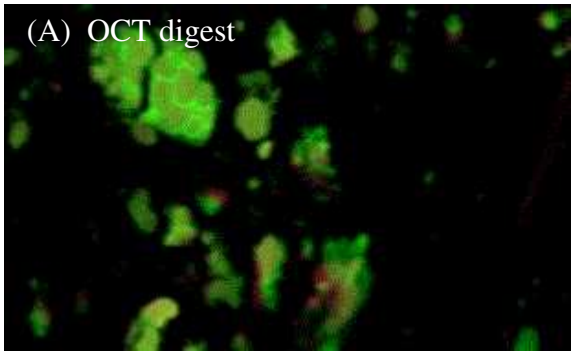
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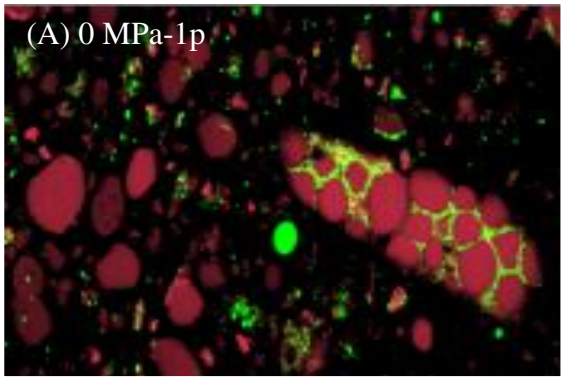
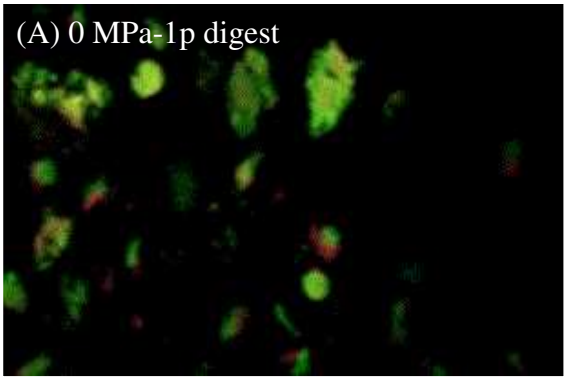
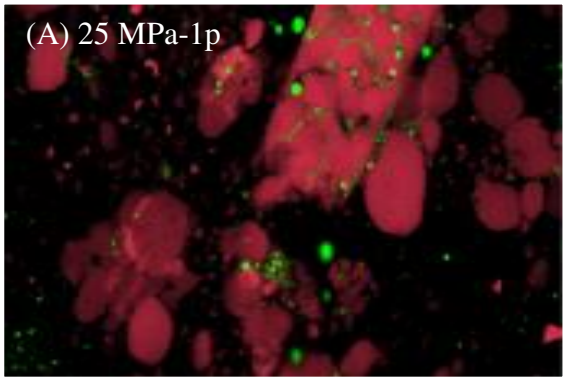
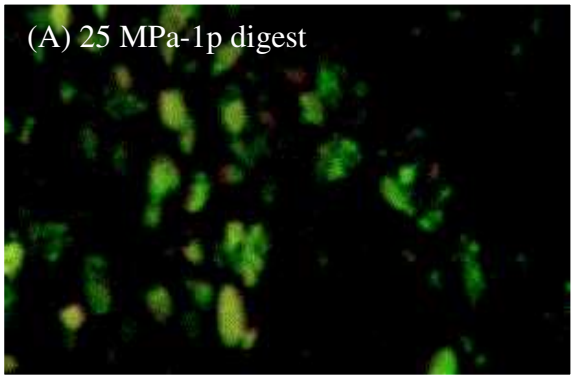

(A) 0 MPa-1p


(A) 0 MPa-1p digest


(A) 25 MPa-1p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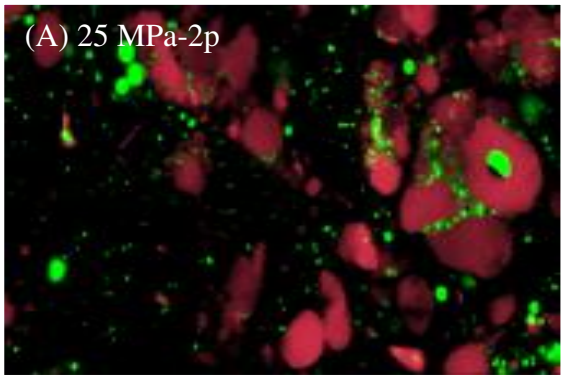
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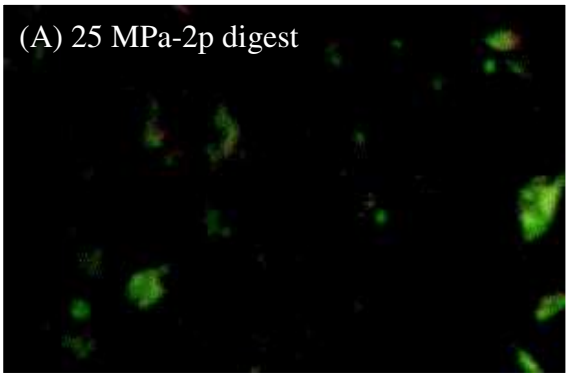
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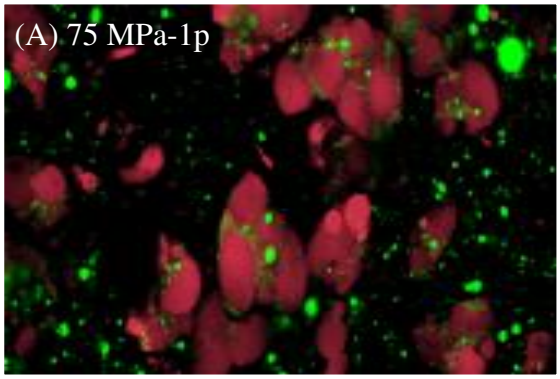
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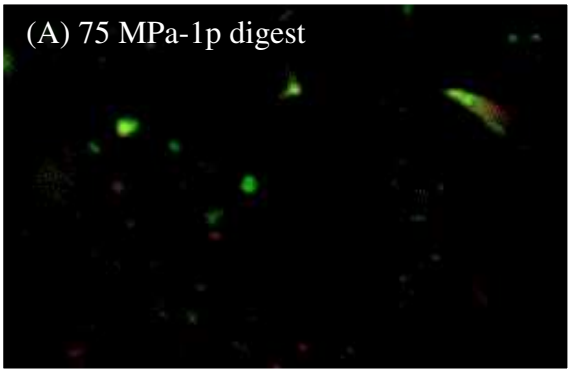
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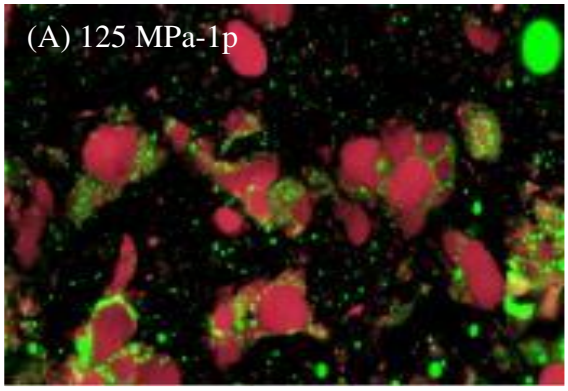
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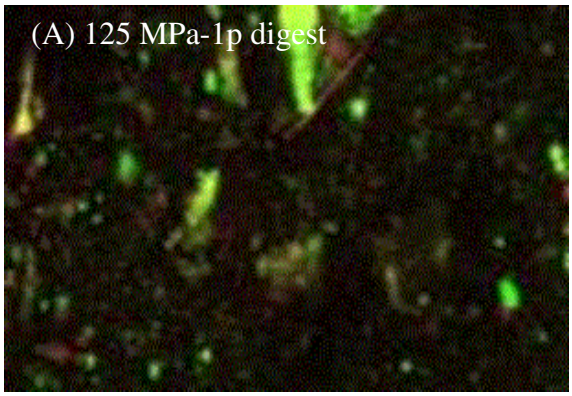
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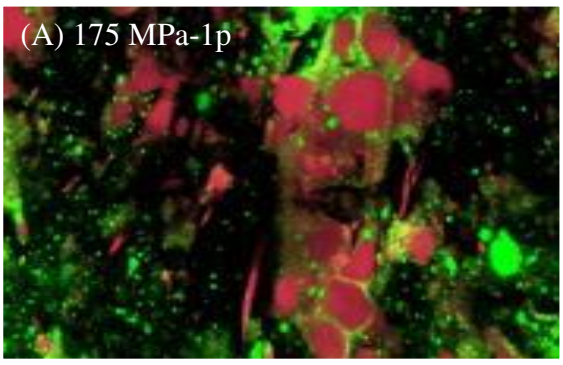
(A) 175 MPa-1p

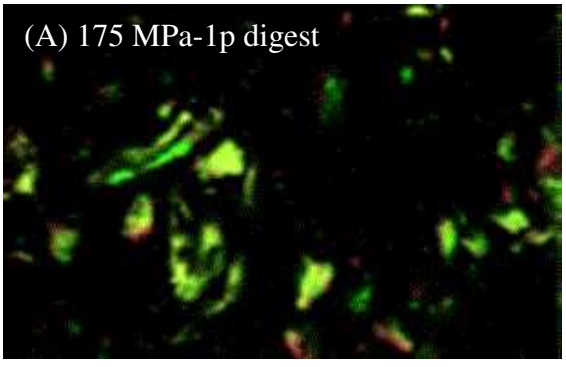
(A) 175 MPa-1p digest

Figure 4.3. Micrographs of HPH-treated okara subjected to different pressure conditions (0, 25, 75, 125, or 175 MPa) and number of passages (1 or 2) and okara digests after gastrointestinal digestion in vitro. Images obtained with confocal microscopy using Fast Green Fast and Nile red dyes, with lipids stained in green and proteins stained in red. (A) visualization of okara; (B) visualization of okara digests. (ICC: intact cotyledon cell; OST: untreated okara; OCT: pre-processed okara; 1p, and 2p corresponded to the number of passages).

4.4. Conclusion

HPH changed the okara microstructure, breaking down cells and fragments, which also led to differences in the microstructure of the digests. The HPH processing led to a higher release of antioxidant compounds and increased solubility and digestibility of okara proteins. In addition, okara contains components with protective action against oxidative damage, which may play a preventive role against chronic diseases. The improvement of the antioxidant activity can represent a technological and bioactive advantage, once the natural antioxidants of okara can act extending the shelf life by preventing lipid oxidation, avoiding the appearance of off-flavors and reduction of nutritional value. Although the natural antioxidants often exhibit a lower antioxidant activity when compared to synthetic antioxidants, they are more advantageous since they do not have limits of use established by law.

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Conflict of interest

The authors declare that there is no conflict of interest.

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CAPÍTULO 5: DISCUSSÃO GERAL

5.1 Discussão geral

A HAP causou intensa mudança na aparência da dispersão de okara, que apresentava consistência heterogênea, com evidentes partículas e nítida separação de fases. A melhoria da consistência de okara foi constatada com o aumento da pressão até 75 MPa e, a pressões mais altas, não houve maiores modificações perceptíveis visualmente. Os resultados de análise de cor mostraram aumento da diferença total de cor (ΔE*) comparando-se amostras homogeneizadas e o controle, sendo constatado um aumento de até cinco vezes. O índice de brancura (WI) também aumentou, e pode ser explicado pelo incremento do número de pequenas partículas que ocasiona maior reflexão da luz (BERNAT *et al*., 2015).

A microestrutura de okara, avaliada por microscopia confocal, também foi modificada pela HAP. Okara possuía células cotiledonares e seus fragmentos, que tiveram a sua presença e tamanho reduzidos com o aumento da pressão. OCT apresentava células típicas da soja, que continha corpos lipídicos e proteicos. Células intactas foram raramente observadas a 25 MPa e estavam ausentes após o tratamento a maiores pressões. A microestrutura da amostra 175 MPa-1p era diferente das demais, com menor dispersão do material, que formava uma estrutura contínua. Isto sugere que a HAP teve um efeito diferente nesta condição de processamento.

O HAP modificou o PDP de quase monomodal observada para OCT para bimodal a partir do tratamento a 25 MPa, com evidente aparecimento de uma região de pequenas partículas. O tamanho das partículas foi reduzido com o aumento da pressão, sendo significantes as reduções nos valores de D [3,2] e D [4,3] até 75MPa-1p e 125 MPa-1p, respectivamente ($p < 0,05$). Comparando-se ao controle OCT, as reduções nos valores de D [3,2] e D [4,3] foram mais acentuadas a 25 MPa-1p. O efeito do uso de múltiplas passagens na redução do tamanho de partículas, foi mais evidente em menores pressões, sob as quais foram obtidos os mesmos efeitos de redução promovidos por maiores pressões com uma passagem. O uso de duas ou três passagens à menores pressões (25 e 75 MPa) levou à reduções de tamanho de partícula observadas a maiores pressões (125 e 175 MPa) usando uma passagem. O impacto de duas passagens foi mais evidente se comparado ao uso de três passagens. O *span* das curvas reduziu com o aumento do número de passagens a 25 MPa, mas o perfil bimodal não foi modificado, o que foi observado somente a partir de 75 MPa, que se tornou quase monomodal. A HAP causou quebras de partículas de okara, pois durante o processamento as estruturas do

material foram forçadas a passer por um espaço estrito e submetidos a cisalhamento, fricção, turbulência, cavitação e fenômenos de impacto intermolecular, seguido de abrupta descompressão (YAN *et al*., 2015). Isto levou a ruptura de células remanescentes e dos seus fragmentos e às mudanças de tamanho e de PDP.

O comportamento reológico, descrito pelo modelo Herschel-Bulkley que apresentou excelente ajuste de dados (R^2> 0,98), forneceu três parâmetros reológicos: tensão inicial (σ_0); índice de consistência do fluido (k) e índice de comportamento do fluido (n). Foram observados efeito da HAP nas curvas de comportamento ao fluxo e nos parâmetros. Comparado ao controle OCT, okara tratado a 25 MPa-1p teve redução de 66% e 85 % em σ_0 e k, respectivamente, sendo estes o mesmos resultados observados a maiores pressões (p> 0,05). O parâmetro n aumentou, respectivamente, 45% e 53% a 25 MPa-1p e 75 MPa-1p quando comparado a OCT, e o mesmo aumento observado a 75 MPa foi observado a maiores pressões (p> 0,05). O uso de múltiplas passagens teve efeito no comportamento ao fluxo e nos parâmetros reológicos. O aumento de 1 para 2 passagens causou redução (65%) em σ_0 a 75 MPa após 2 passagens e aumento no parâmetro n (13-21%) (p<0,05) até 25 MPa, (p <0,05). O aumento de 2 para 3 passagens modificou apenas o parâmetro n, aumentando-o apenas a 25 MPa-1p (~ 15%) (p <0,05). A HAP levou à grandes reduções dos parâmetros k e σ_0 e aumento de n, tornando o fluido próximo do comportamento Newtoniano. As mais acentuadas mudanças nos parâmetros σ_0 e k foram observadas a 25 MPa e em n a 75 MPa. O uso de múltiplas passagens mudou os parâmetros reológicos, sendo mais evidentes as alterações à menores pressões e com o uso de duas passagens.

Não foi observado mudança no perfil de fibras de okara em função da pressão de homogeneização. Apesar da alta pressão ser descirta como uma tecnologia que pode modificar o perfil de fibras dos alimentos (HUANG *et al*. 2015; MATEOS-APARICIO *et al*., 2010; ULBRICH; FLÖTER, 2014), o que pode afetar a funcionalidade dos produtos (TEJADA-ORTIGOZA *et al*., 2015).

As proteínas de okara, avaliadas por SDS-PAGE em condições redutoras, mostraram o mesmo perfil descrito para as proteínas de soja, não sendo modificado por nenhuma condição de pressão ou número de passagens. A solubilidade proteica em água de okara submetido a uma passagem foi avaliada, exceto para a amostra 25 MPa que teve a solubilidade determinada também com a aplicação de duas passagens. A solubilidade aumentou com o pré-tratamento e com a HAP, sendo crescente em função da pressão de homogeneização aplicada (p<0,05). Okara apresentou baixa solubilidade proteica, o que é esperado, já que é um

resíduo resultante da extração de proteínas solúveis (VISHWANATHAN; SINGH; SUBRAMANIA, 2011). A máxima solubilidade das proteínas de okara foi a 175 MPa, 90% superior à apresentada por OCT. O aumento de uma para duas passagens a 25 MPa causou incremento de solubilidade de 21%. O aumento da solubilidade proteica deve-se ao aumento da extração de proteínas, pois com o rompimento de células de cotilédone e de seus fragmentos houve a liberação de proteínas antes aprisionadas em estruturas celulares, em agregados insolúveis ou em interações com outros componentes. Preece *et al*. (2015) destacaram que a principal barreira para extração de componentes da soja é a parede celular, que possui uma rede robusta, difícil de comprimir (PREECE *et al*., 2015), além do efeito também da insolubilidade dos componentes e do aprisionamento no resíduo. O aumento da solubilidade das proteínas observado neste estudo está em consonância com outros estudos que utilizaram alta pressão para liberar proteínas de grãos de soja (OMI *et al*., 1996) e para solubilizar componentes de okara (PORFIRI *et al*., 2017).

A presença de fatores antinutricionais também foi avaliada por meio da determinação da atividade ureática (AU). A AU da soja e do okara foram, respectivamente, 2,05±0,04 e 0,02±0,02, o que indica que o tratamento térmico empregado no processamento do EHS foi suficiente para reduzir os fatores antinutricionais a níveis adequados no okara (WACHIRAPHANSAKUL; DEVAHASTIN, 2007; WACHIRAPHANSAKUL; DEVAHASTIN, 2005). Portanto, os fatores antinutricionais que são conhecidos por possuir a capacidade de inibir a digestão proteica (MONTEIRO *et al*., 2004), possivelmente não alteraram a digestibilidade das proteínas de okara.

A digestibilidade das proteínas de okara foi alterada positivamente pela HAP. A amostra tratada a 25 MPa teve a sua digestibilidade aumentada em 24% e 12% ao fim da DG e da DI, respectivamente, em relação a OCT ($p<0,05$). O mesmo comportamento também foi verificado nas maiores pressões estudadas ($p<0,05$).

As características dos digeridos foram estudadas por meio da avaliação do perfil de distribuição de MM por CLAE-EM e pela visualização da microestrutura por microscopia confocal. Foram constatados que os controles OST e OCT possuíam apenas peptídeos menores que 1,4 kDa e todos os demais digeridos apresentaram, majoritariamente, peptídeos nesta faixa de tamanho. Além dos peptídeos menores que 1,4 kDa, o digerido 0 MPa possuiu também peptídeos entre 1,4 e 3,5 kDa, os digeridos 25 MPa-1p, 25 MPa-2p, 75 MPa, 125 e 175 MPa apresentaram peptídeos entre 6,5 e 14,4 kDa, sendo que o digerido 175 MPa também apresentou peptídeos entre 3,5 e 14,4 kDa.

A microestrura dos digeridos foi observada com o objetivo de avaliar a possível relação entre alterações de microestrutura e as diferenças observadas na digestibibilidade proteica e no perfil de MM dos peptídeos dos digeridos. Células de cotilédone intactas (CCI) foram visualizadas nos digeridos das amostras controles (OST, OCT e 0 MPa), mas não nas amostras homogeneizadas. Não foram observadas diferenças nas microestruturas de okara tratado a 25, 75 e 125 MPa, exceto pela rara presença de CCI nas amostras submetidas a 25 MPa. A amostra tratada a 175 MPa-1p apresentou característica microestrutural diferente das demais, com materiais menos dispersas, formando uma estrutura contínua. Ao comparar as micrografias de amostras homogeneizadas com os seus respectivos digeridos, notou-se o mesmo perfil de microestrutura entre amostra e digerido, com a diferença que menos material proteico foi observado nos digeridos. Isto sugere que a microestrutura de okara, modificada pela HAP, influenciou a digestão das proteínas e, portanto, as observações microscópicas foram coerentes com os resultados de digestibilidade proteica e com o perfil de MM dos peptídeos. A diferença no perfil de MM, aliada a diferença de microestrutura observada para a amostra tratada a 175 MPa sugerem que a HAP modificou de maneira diferente a microestrutura de okara e, isso levou a uma diferença no perfil de peptídeos gerados após a simulação da digestão gastrointestinal *in vitro*. A estrutura mais contínua, menos espalhada, recoberta por gotículas de lipídeos pode ter contribuído para modificar a acessibilidade enzimática e, como consequência ter um perfil de peptídeos diferentes das demais amostras.

O efeito da HAP na atividade antioxidante neste estudo foi investigado pela determinação de SRRFC, FRAP e ORAC. A HAP causou aumento nos teores de SRRFC e na atividade antioxidante determinada por FRAP ($p<0,05$), mas não na determinada por ORAC ($p>0,05$). Em relação à OCT, o máximo aumento no teor de SRRFC (66%) e no valor de FRAP (38%) foi observado a 75 MPa e, estes percentuais foram os mesmos observados a pressões mais elevadas. Valores de SRRFC e de FRAP não foram alterados pelo aumento do número de passagens à 25 MPa ($p>0,05$). O fato dos valores de ORAC não terem sido alterados e os de FRAP e SRRRF terem aumentado em função do aumento da pressão indica que os componentes responsáveis pelo aumento da atividade antioxidante atuaram predominantemente por mecanismo de doação de elétrons. Silva *et al*. (2017) relatam em pesquisa sobre atividade antioxidante em linhaça que proteínas atuam como antioxidantes principalmente pela transferência de hidrogênio e que os compostos fenólicos, além deste mecanismo, atuam também pela doação de elétrons. Portanto, possivelmente os compostos fenólicos solúveis (presentes nos vacúolos das células) e insolúveis (encontrados nas paredes celulares ou em

associação com outros compostos) (VONG; LIM; LIU, 2017; PANDEY; RIZVI, 2009) foram liberados com o pré-tratamento e, principalmente, com o uso da HAP. A redução do tamanho das partículas de okara causou a liberação de componentes como proteínas e peptídeos (WIBOONSIRIKUL *et al*., 2013), compostos fenólicos (RASHAD *et al*., 2011) e outros tais como α-tocoferol, γ-tocoferol, δ-tocoferol (MATSUO, 1997), coumestanos, saponinas, fitatos (LI *et al*., 2012), que podem ter contribuído para o aumento do teor de SRRFC e/ou da atividade antioxidante.

Os resultados indicam que a HAP causou rompimento de estruturas celulares de okara e de seus fragmentos, o que levou a redução do tamanho de partículas e a maior extrabilidade dos componentes de okara, como proteínas e compostos com atividade antioxidante antes encapsulados dentro de paredes celulares (BHATTARAI *et al*., 2018; SINGH; GALLIER, 2014). Pode ter ocorrido também o rompimento de interações entre proteínas e outros componentes como fibras e outros polissacarídeos (FAFAUNGWITHAYAKUL; HONGSPRABHAS; HONGSPRABHAS, 2011; TURGEON; RIOUX, 2011). As mudanças observadas nos parâmetros reológicos, especialmente a redução do índice de consistência, podem ser explicadas pela redução do tamanho de partícula e aumento da solubilidade proteica. As pequenas partículas podem preencher o espaço entre as partículas maiores levando a um efeito "lubrificante", reduzindo assim a resistência ao fluxo e alterando o índice de consistência (SERVAIS *et al*., 2002). Além disso, partículas pequenas requerem menos energia para fluir, o que pode explicar o comportamento do fluxo de okara após o processamento de HAP. Além da redução do tamanho de partícula, a maior solubilização de proteínas também pode ter contribuído para a redução da consistência. A dispersão aquosa de okara pode ser descrita de maneira similar ao modelo para sucos de frutas proposto por Augusto; Ibarz; Cristianini (2012a), como uma matriz composta de duas fases, uma dispersa e o soro. A fase dispersa é formada de células de tecido da soja, seus fragmentos e componentes insolúveis, enquanto o soro é uma solução aquosa contendo os componentes solúveis de okara. Com a ruptura de células e de seus fragmentos devido a homogeneização, além do aumento da área superficial das partículas, há liberação e exposição de constituintes celulares (AUGUSTO; IBARZ; CRISTIANINI, 2012b), como proteínas. Com o aumento da solubilização proteica houve redução na fração de células, fragmentos e componentes insolúveis. Nossos achados estão de acordo com outros autores, que relataram que as alterações reológicas proporcionadas pela redução do tamanho das partículas por HPH melhoraram as características físicas das

matrizes das plantas (AUGUSTO *et al*., 2013; KUBO *et al*., 2013; LEITE *et al*., 2015; LEITE *et al*., 2014; YAN *et al*., 2015).

A maior extrabilidade de proteínas possivelmente facilitou a hidrólise destes componentes pelas enzimas digestivas, aumentando, então, sua bioacessibilidade. Porém, mesmo com a parede celular rompida, a incompleta digestão das proteínas de okara foi observada e isso pode estar relacionada à presença de fibras nas amostras, devido a interações entre estes componentes, o que pode afetado a suscetibilidade à ação das enzimas (BHATTARAI *et al*., 2017). Portanto, os achados desta pesquisa mostram como a HAP pode alterar a microestrutura de okara e isso ocasiona uma melhoria de suas características físicas, nutricionais e de seu potencial bioativo, tornando-o um material com melhores propriedades para ser manipulado e transformado na indústria e, possivelmente, proporcionando melhores benefícios ao ser consumido.

5.2. Referências bibliográficas

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CAPÍTULO 6: CONCLUSÃO GERAL

Nesta pesquisa, a HAP, mesmo a baixas pressões, mostrou ser uma alternativa tecnológica eficiente para transformar uma dispersão aquosa de okara em um material com textura melhorada, mais fácil de ser incorporado em sistemas alimentícios e de ser manipulado em operações de bombeamento e mistura, por exemplo.

Com a redução da consistência de okara, possivelmente, também poderá haver a possibilidade de transformá-lo em material desidratado por meio de tecnologia que exija a sua fluidização, como em spray dryer, o que não seria possível em sua forma original. Este material, rico em fibras e proteínas, além de possuir melhor característica física, teve a sua propriedade nutricional e potencial bioativo positivamente afetados, com aumento da atividade antioxidante e ligeiro incremento da digestibilidade proteica *in vitro.*

O aproveitamento de okara utilizando a tecnologia de HAP poderá tornar possível o processamento mais sustentável do EHS, com uso eficiente da soja, e ainda permitirá a transformação de um resíduo em um ingrediente que preservará as características naturais da matéria prima devido a tecnologia escolhida.

Na indústria de alimentos, criar estratégias para aproveitamento de resíduos representa uma oportunidade para que elas cresçam economicamente, ao mesmo tempo em que se ajustam a um novo paradigma de produção e consumo que considera fundamental o uso otimizado dos recursos naturais. Portanto, o aproveitamento de resíduos gerados na indústria de extrato hidrossolúvel de soja, como o okara, é importante. No entanto, embora seja rico do ponto de vista nutricional e possua potencial bioativo, este subproduto é descartado ou destinado à alimentação animal. O seu uso em alimentação humana é restrito devido, principalmente, a textura desagradável que proporciona aos alimentos e a dificuldade de transformá-lo devido a sua alta coesividade.

Dentre as diretrizes nacionais estabelecidas pelo Governo Federal para promover o conhecimento científico e tecnológico do Brasil, destaca-se a defesa do meio ambiente e a garantia do desenvolvimento sustentável. Para alcançar este resultado é necessário adotar estratégias que contribuam com a eliminação ou redução dos impactos decorrentes das atividades industriais. No contexto da produção agrícola e de alimentos, a inclusão ou adequação de procedimentos que permitam a reutilização de resíduos permite não somente reduzir a poluição gerada nos processos, como também os custos decorrentes do seu tratamento, além de até mesmo proporcionar a geração de receitas, provocando um fator de competitividade para as indústrias.

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APÊNDICES

APÊNDICE A. Tamanho médio de partículas de okara homogeneizado sob diferentes condições de pressão (0, 25, 75, 125 ou 175 MPa) e de número de passagens (1, 2 ou 3 passagens).

Tratamento	D[3,2]	D[4,3]
OST	235.7±20.9921 (A)	664.3±24.5977 (A)
OCT	98.9±15.8456 (B) (a)	411.9±16.9479 (B) (a)
0-1p	73.1±4.5007 (b)	358.0±15.0590 (b)
0-2p	72.0±0.5067 (b)	346.2±17.6310 (c)
0-3p	63.9±3.1286 (c)	328.6±13.9664 (d)
25-1p	35.6±1.9515 (d)	100.0±3.6814 (e)
25-2p	23.0±0.5067 (e)	60.1±0.8223 (f)
25-3p	20.9±0.4826 (f,g)	49.5±0.9773 (f,g)
75-1p	21.8±0.4809 (f)	51.2±1.0663 (f)
75-2p	17.9±0.6861 (f,g)	32.0±2.2338 (h,i)
75-3p	16.0±0.3935 (f,g)	24.8±1.6368 (i)
125-1p	20.0±0.6622 (f,g)	39.5±1.4740 (g,h)
125-2p	15.5±0.1962 (g)	24.0±1.6763 (i)
125-3p	16.1± (f,g)	21.4±0.3510 (i)
175-1p	17.5± (f,g)	32.4±2.5236 (h,i)
175-2p	19.9± (f,g)	26.7±3.0481 (i)
175-3p	16.1± (f,g)	21.9±1.8194 (i)

(OST: okara não pré-processado; OCT: okara pré-processado; 1p, 2p e 3p correspondem ao número de passagens; todas as amostras homogeneizadas foram pré-processadas).

APÊNDICE B. Parâmetros reológicos, a 25°C, de okara homogeneizado sob diferentes condições de pressão (0, 25, 75, 125 ou 175 MPa) e número de passagens (1, 2 ou 3 passagens) obtidos com o modelo Herschel-Bulkley.

Tratamento	n° p	σ_0 (Pa)	k (Pa·s^n)	n	R^2	Modelo
OCT	-	30.14 ± 7.95 (b)	25.74 ± 5.80 (a)	0.27 ± 0.07 (i)	0.9830	$\sigma = 26.56 + 28.74 \cdot \dot{\gamma}^{0.26}$
0 MPa	1	45.36 ± 11.33 (a)	22.12 ± 10.76 (a,b)	0.34 ± 0.10 (h,i)	0.9910	$\sigma = 38.67 + 27.47 \cdot \dot{\gamma}^{0.31}$
	2	43.07 ± 14.63 (a)	16.93 ± 2.73 (b,c)	0.38 ± 0.04 (g,h)	0.9950	$\sigma = 10.23 + 3.78 \cdot \dot{\gamma}^{0.49}$
	3	48.42 ± 2.57 (a)	11.91 ± 1.15 (c)	0.43 ± 0.01 (f,g)	0.9990	$\sigma = 48.42 + 11.91 \cdot \dot{\gamma}^{0.43}$
25 MPa	1	10.23 ± 2.06 (c,d,e)	3.78 ± 1.14 (d)	0.49 ± 0.03 (f)	1.000	$\sigma = 10.23 + 3.78 \cdot \dot{\gamma}^{0.49}$
	2	12.51 ± 2.99 (c,d,e)	1.71 ± 0.60 (d)	0.57 ± 0.03 (e)	1.000	$\sigma = 12.51 + 1.71 \cdot \dot{\gamma}^{0.57}$
	3	5.38 ± 1.46 (d,e)	0.49 ± 0.18 (d)	0.68 ± 0.03 (c,d)	0.9999	$\sigma = 5.38 + 0.49 \cdot \dot{\gamma}^{0.68}$
75 MPa	1	16.78 ± 0.67 (c)	1.63 ± 0.06 (d)	0.58 ± 0.01 (e)	0.9998	$\sigma = 16.78 + 1.63 \cdot \dot{\gamma}^{0.58}$
	2	5.90 ± 0.71 (d,e)	0.26 ± 0.02 (d)	0.71 ± 0.00 (a,b,c)	0.9995	$\sigma = 5.90 + 0.26 \cdot \dot{\gamma}^{0.71}$
	3	3.07 ± 0.16 (e)	0.13 ± 0.01 (d)	0.77 ± 0.01 (a,b)	0.9998	$\sigma = 3.07 + 0.13 \cdot \dot{\gamma}^{0.77}$
125 MPa	1	12.94 ± 0.34 (c,d)	0.94 ± 0.03 (d)	0.61 ± 0.01 (d,e)	0.9998	$\sigma = 12.94 + 0.94 \cdot \dot{\gamma}^{0.61}$
	2	5.18 ± 0.42 (d,e)	0.25 ± 0.02 (d)	0.72 ± 0.01 (a,b,c)	0.9996	$\sigma = 5.18 + 0.25 \cdot \dot{\gamma}^{0.72}$
	3	4.80 ± 0.30 (d,e)	0.18 ± 0.01 (d)	0.78 ± 0.01 (a)	0.9999	$\sigma = 4.80 + 0.18 \cdot \dot{\gamma}^{0.78}$
175 MPa	1	13.25 ± 0.90 (c,d)	0.76 ± 0.04 (d)	0.62 ± 0.01 (d,e)	0.9993	$\sigma = 13.25 + 0.76 \cdot \dot{\gamma}^{0.62}$
	2	7.75 ± 0.97 (c,d,e)	0.39 ± 0.04 (d)	0.71 ± 0.02 (b,c)	0.9998	$\sigma = 7.75 + 0.39 \cdot \dot{\gamma}^{0.71}$
	3	7.76 ± 0.49 (c,d,e)	0.34 ± 0.03 (d)	0.73 ± 0.01 (a,b,c)	0.9999	$\sigma = 7.76 + 0.34 \cdot \dot{\gamma}^{0.73}$

(OST: okara pré-processado; OCT: okara pré-processado; 1p, 2p e 3p correspondem ao número de passagens; todas as amostras homogeneizadas foram pré-processadas).