ALLINE ARTIGIANI LIMA TRIBST

EFFECTS OF HIGH PRESSURE HOMOGENIZATION IN THE ACTIVITY AND STABILITY OF COMMERCIAL ENZYMES

"*EFEITO DA HOMOGENEIZAÇÃO À ALTA PRESSÃO NA ATIVIDADE E ESTABILIDADE DE ENZIMAS COMERCIAIS*"

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Orientador: Prof. Dr. Marcelo Cristianini

"*EFEITO DA HOMOGENEIZAÇÃO Á ALTA PRESSÃO NA ATIVIDADE E ESTABILIDADE DE ENZIMAS"*

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Prof. Dr. Marcelo Cristianini

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“Ensinar não é transferir conhecimento, mas criar possibilidades para a sua produção ou a sua construção. Quem ensina aprende ao ensinar e quem aprende ensina ao aprender.”

Paulo Freire

Aos meus pais, JOSÉ e MARIA OLÍVIA;
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Resumo Geral

A homogeneização à alta pressão (HAP) é uma operação unitária capaz de alterar a conformação e, consequentemente a funcionalidade de polissacarídeos, proteínas e enzimas. O objetivo deste trabalho foi avaliar o efeito da HAP na atividade e estabilidade de cinco enzimas comerciais com aplicação na indústria de alimentos (α-amilase de *Aspergillus niger*, protease neutra de *Bacillus subtilis*, β-galactosidase de *Kluyveromyces lactis*, amiloglicosidase de *A. niger* e glicose oxidase de *A. niger*). Para cada enzima, a atividade foi avaliada antes e após a HAP (até 200 MPa) em diferentes temperaturas e pH. Além disso, a reversibilidade dos efeitos do processo foi determinada indiretamente através da medida de atividade da enzima após um período de repouso. Os resultados de α-amilase demonstraram que a enzima é altamente estável ao processo de HAP (em pressões de até 150 MPa), independentemente do pH e temperatura de processo e da ausência de cálcio no tampão de diluição da enzima. Os resultados da β-galactosidase, por outro lado, mostraram que a enzima é pouco estável, apresentando redução da atividade (~30%) após HAP a 150 MPa quando processadas em pH não ótimo para atividade da enzima. Os resultados obtidos para a protease neutra, amiloglicosidase e glicose oxidase indicaram que o efeito da HAP foi dependente dos parâmetros utilizados no processo (pH, temperatura e pressão de homogeneização) e das condições utilizadas na medida de atividade (pH, temperatura e tempo de estocagem). Para estas três enzimas, significativos ganhos de atividade e/ou estabilidade foram observados para pelo menos uma das condições avaliadas, sendo que os mais importantes foram: (i) redução da temperatura ótima de atividade da protease neutra de 55 para 20ºC após HAP a 200 MPa, (ii) aumento da atividade da glicose-oxidase à 75ºC após HAP a 150 MPa, (iii) aumento da atividade residual entre 100 e 400% após armazenamento refrigerado de glicose-oxidase homogeneizada em diferentes pressões, (iv) aumento da atividade de amiloglicosidase à 80ºC após a HAP a 100 MPa. A reversibilidade das alterações observadas foi inferida pela avaliação da atividade da enzima após um período de repouso, sendo as alterações determinadas como reversíveis (protease neutra, glicose-oxidase, amiloglicosidase) ou irreversíveis

(protease neutra, glicose-oxidase, β-galactosidase) em função dos parâmetros de processo. O efeito de processamentos sequenciais sobre a glicose oxidase, a protease e a amiloglicosidase também foi avaliado e os resultados demonstraram que, para a maioria das condições estudadas, a atividade da enzima se manteve igual à obtida após o primeiro ciclo de homogeneização ou apresentou uma redução. Uma exceção foram os resultados da glicose oxidase homogeneizada a 150 MPa por 3 vezes, que apresentou aumento de atividade de aproximadamente 150% em relação à enzima nativa. A partir dos resultados, conclui-se que o efeito da HAP é diferente para cada enzima e que as maiores alterações ocorrem em condições de atividade não ótima e para enzimas de estruturas mais complexas, como é o caso da glicose oxidase. Os resultados obtidos apresentam aplicação direta, para modificação e melhoria do desempenho de enzimas comerciais, e preenchem uma lacuna científica importante sobre o conhecimento dos efeitos do processo de HAP em enzimas.

Palavras-chave

Homogeneização à alta pressão, processo não térmico, ativação enzimática, alterações na conformação de enzimas.

Summary

High pressure homogenization (HPH) is a unitary operation capable to alter the conformation and, consequently, the functionality of polyssacharides, proteins and enzymes. This work aimed to study the HPH effects on activity and stability of five commercial enzymes intensively applied in food industry (α-amylase from *Aspergillus niger*, β-galactosidase from *Kluyveromyces lactis*, neutral protease from *Bacillus subtilis*, amyloglucosidase from *A. niger* and glucose-oxidase from *A. niger*). The activity of each enzyme was studied before and after HPH process (up to 200 MPa) at different temperatures and pH. Moreover, the process reversibility was indirectly determined by the activity measured after a rest period under refrigeration (8ºC).The results revealed that α-amylase was highly stable to HPH up to 150 MPa, independent on the pH or temperature used in the HPH process or the presence of calcium in buffer. On the other hand, the results of β-galactosidase indicated that enzyme was partially inactivated (~ 30%) after homogenization at 150 MPa when processed at non optimum pH. The HPH effects on neutral protease, amyloglucosidase (AMG) and glucose oxidase (GO) were dependent on the process parameters (pH, temperature and homogenization conditions) and the activity measurement conditions (pH, temperature and storage time). For these enzymes, it was observed activity and/or stability improvement after some process. The main improvements were: (i) change of the optimum temperature of neutral protease from 55 to 20ºC after HPH at 200 MPa, (ii) improvement of GO activity at 75ºC after HPH at 150 MPa, (iii) enzyme activity improvement between 100 and 400% after GO refrigerated storage, (iv) improvement on amyloglucosidase activity at 80ºC after HPH at 100 MPa. The reversibility of the HPH effects was evaluated after a rest period. The reversibility was dependent on the process parameters; but, in general, neutral protease, GO and AMG were reversible, while the results of neutral protease, GO and β-galactosidase were irreversible. The effects of sequential homogenization processes (sequential passes) on GO, AMG and neutral protease were evaluated and the results showed that the enzyme activity remained equal or reduced after 2 or 3 cycles of homogenization. An exception was the result obtained for GO

homogenized at 150 MPa, which showed an activity improvement of 150% after three passes. The results evaluation of this research showed that HPH effects on enzymes were different for each enzyme. The main alterations occured at non optimum condition of enzyme activity and for enzymes with complex structure as the GO. The obtained results can be directly applied for improvement of enzyme industrial production. Also, the results enriched the scientific knowledge about the HPH effects on enzymes.

Keywords

High pressure homogenization, non-thermal processing, enzyme activation, enzymes conformational changes.

Introdução e Justificativas

A homogeneização à alta pressão foi estudada por diversos autores como metodologia não térmica para a conservação de alimentos, apresentando resultados semelhantes ao processo de pasteurização térmica. Nos últimos anos, algumas pesquisas começaram a descrever o efeito da HAP sobre macromoléculas, como polissacarídeos e proteínas. Os resultados mostraram que o processo é capaz de promover alterações na conformação destas moléculas, chegando à quebra de ligações covalentes dependendo da pressão de homogeneização e do tipo de estrutura avaliada. Essas alterações conformacionais refletem em alterações nas propriedades funcionais das moléculas.

Enzimas são proteínas com funções catalíticas que podem ser encontradas naturalmente em alimentos ou adicionadas intencionalmente para o desempenho de alguma função catalítica específica, como hidrólise de amidos e proteínas, visando à obtenção de produtos com diferentes aplicações e funcionalidades. Apesar da alta qualidade dos produtos obtidos por ação enzimática, o emprego de enzimas na indústria de alimentos apresenta duas importantes barreiras, que são o alto custo das enzimas e a baixa estabilidade em determinadas condições de processo (pH, temperatura, meio reativo).

Recentemente, foram iniciados alguns trabalhos para avaliar o efeito da HAP em enzimas, partindo do objetivo de inativar enzimas reconhecidamente indesejáveis por causarem escurecimento, *off flavor* e separação de fases em alguns alimentos. Alguns destes trabalhos, entretanto, destacaram que o processo causou a ativação ou estabilização das enzimas, devido a alterações nas estruturas das mesmas.

Considerando a necessidade de redução de custo e aumento de estabilidade para melhor viabilização da aplicação de enzimas na indústria de alimentos e os resultados obtidos para algumas enzimas submetidas ao processo de homogeneização à alta pressão, a justificativa para a realização deste trabalho foi a observação da possibilidade real de aplicação da HAP como uma operação unitária para melhorar o desempenho de enzimas comerciais.

Considerando que poucos trabalhos haviam sido conduzidos até o momento inicial da pesquisa para avaliação desta tecnologia em enzimas, optou-se pela realização de um estudo de base sobre o efeito do processo em enzimas de

importância comercial para a indústria de alimentos. As enzimas escolhidas (α-amilase, amiloglicosidase, β-galactosidase, glicose oxidase e protease neutra) são de grande relevância na produção de alimentos ou de ingredientes de alimentos e apresentam um volume apreciável das vendas.

Para estas enzimas, os estudos foram realizados mediante avaliação da atividade após a homogeneização em diferentes pressões e utilizando-se diferentes condições de processos (temperatura e pH). A avaliação da atividade após a estocagem permitiu uma inferência sobre a estabilidade da enzima homogeneizada. Para aquelas enzimas que apresentaram as modificações de maior relevância, foi estudado também o efeito de processamentos sequenciais (múltiplos passes) de forma a avaliar se os ganhos observados para um processo único seriam aumentados com os processamentos sequenciais.

Os ensaios preliminares e de definção dos métodos analíticos utilizados são descritos no capitulo 2. Os efeitos do processamento a alta pressão sobre cada uma das enzimas são apresentados nos capítulo 3-7 e o capítulo 8 indica os resultados obtidos para múltiplos passes.

Capítulo 1. Revisão Bibliográfica e Objetivos

1.1. Revisão Bibliográfica

1.1.1. Enzimas

Enzimas são proteínas globulares (DOBLE, KRUTHIVENTI, GAIKAR, 2004) produzidas por organismos vivos e apresentam a função de catalisar reações bioquímicas necessárias para a sobrevivência dos mesmos (OLEMPSKA-BEER et al., 2006). As enzimas são subdivididas em oxidoredutases, transferases, hidrolases, liases, isomerases e ligases, sendo que esta divisão baseia-se nas reações catalisadas (WHITAKER, 2002; DOBLE, KRUTHIVENTI, GAIKAR, 2004). A maior vantagem da aplicação de enzimas é a sua especificidade na reação com determinado substrato (mecanismo conhecido como chave-fechadura) com consequente obtenção de produtos bem conhecidos (WHITAKER, 2002). A reação enzimática ocorre pela formação do complexo enzima-substrato através de pontes de hidrogênio e interações de Van der Walls, e posterior dissociação, com a liberação do produto e da enzima em sua forma nativa (DOBLE, KRUTHIVENTI, GAIKAR, 2004)

Todos os organismos vivos são produtores de enzimas. Entretanto, apenas 8% da produção de enzimas comerciais provêm da extração de animais e 4% de plantas, sendo o restante obtido a partir de fermentação microbiana (ARAPOGLOU, LABROPOULOS, VARZAKAS, 2009). Essa preferência pela utilização de enzimas microbianas é explicada pelos seguintes fatores (ARAPOGLOU, LABROPOULOS, VARZAKAS, 2009):

1. Baixo custo de produção,
2. Maior previsibilidade de ação e, portanto, maior facilidade no controle do processo,
3. Utilização de matérias primas de composição constante,
4. Atividade constante, não sendo afetada pelos efeitos sazonais do clima e disponibilidade de alimentos (como é o caso das enzimas animais e vegetais),
5. Opções de enzimas com estabilidades variadas em diferentes condições de processo (pH, temperatura).

As enzimas são aplicadas industrialmente no processo produtivo de diversos alimentos como leites, queijos, gorduras, produtos de panificação, cerveja e outros alimentos fermentados, sucos e outros produtos a base de frutas, rações, farinhas e gelatinas (HAKI, RAKSHIT, 2003; KRAJEWSKA, 2004; JAYANI, SAXENA, GUPTA, 2005). Além disso, podem ser utilizadas na fabricação de papel, de couro e no tratamento de águas residuárias (HAKI, RAKSHIT, 2003; JAYANI, SAXENA, GUPTA, 2005), nas áreas médica (KRAJEWSKA, 2004), farmacêutica, e nas indústrias têxtil e de detergentes (IYER, ANANTHANARAYAN, 2008). O mercado mundial de venda de tem perspectiva de movimentar 8 bilhões em 2015 (FREEDONIA, 2012). Exemplos de enzimas comerciais importantes e com grande aplicação na área de alimentos são: proteases, α-amilase, amiloglicosidase, glucose oxidase e β-galactosidase. Maiores detalhes sobre mecanismo de ação e aplicações destas enzimas são descritos a seguir.

A α-amilase (1,4-α-D-glucano glucohidrolase, EC 3.2.1.1) é uma endoglucanase que catalisa arbitrariamente a hidrólise das ligações glicosídicas α-(1,4) internas de amidos, dextrinas e oligossacarídeos (WONG, ROBERTSON, 2003). Estruturalmente, a α-amilase é uma metaloenzima que contém pelo menos um íon de cálcio divalente em sua estrutura, o qual, segundo alguns autores apresenta um papel importante na estabilidade da enzima (ROBYT, FRENCH, 1963; VIOLET, MEUNIER, 1989; HMIDET et al., 2010). Esta enzima apresenta alto valor comercial, detendo a maior fatia de mercado de enzimas para aplicação na indústria de amido e panificação (WONG, ROBERTSON, 2003; GUPTA, GUPTA, RATHI, 2004) e é também utilizada na produção de etanol, detergentes e na indústria têxtil (WONG, ROBERTSON, 2003). Mais recentemente foi desenvolvida a aplicação da α-amilase para redução de consistência em sucos pela hidrólise do amido, o qual pode estar presente tanto por ser característico das frutas como por ser oriundo de frutas imaturas misturadas às maduras, que passam despercebidas devido ao grande volume de suco processado pelas indústrias (CECI, LOZANO, 2002; ZHANG, WANG, XU, 2007; CHEIRSILP, UMSAKUL, 2008; DOMINGUES et al., 2011).

A amiloglicosidase (AMG) ou glicoamilase (1,4-a-D-glucano glucohidrolase, E.C. 3.2.1.3) hidrolisa sucessivamente ligações glicosídicas α-1,4 e α-1,6 a partir das

extremidades não redutoras de amido e dextrinas, produzindo glicose (REILLY, 2003; ADENIRAN, ABIOSE, OGUNSUA, 2010). Estruturalmente, a AMG é classificada em seis grupos diferentes, sendo o tipo predominante o que contém três regiões distintas e dois grupos globulares funcionais (KUMAR, SATYANARAYANA, 2009). A principal aplicação da AMG é a sacarificação do amido, visando a aplicação em indústrias de alimentos como adoçante ou a obtenção de matéria prima para produção de etanol de alta qualidade, a ser utilizado na produção de perfumes, remédios e bebidas alcoólicas (ZANIN, MORAES, 1998; KUMAR, SATYANARAYANA, 2009). Adicionalmente, sua aplicação em sucos tem crescido (RIBEIRO et al., 2010) tendo-se os mesmos objetivos da aplicação da α-amilase.

A glicose oxidase (β-D-glicose:oxigênio 1-oxidoredutase, EC 1.1.3.4) catalisa a oxidação de β-D-glicose em ácido glucônico utilizando o oxigênio molecular como um aceptor de elétrons com produção simultânea de peróxido de hidrogênio (FIEDUREK, GROMADA, 1997; BANKAR et al., 2009). Estruturalmente, a glicose oxidase é uma glicoproteína dimérica, contendo duas cadeias de polipeptídeos que são unidas através de pontes de sulfeto e aproximadamente 16% de açúcares (glicose, manose e hexomanose) (BANKAR et al., 2009). É reconhecidamente uma enzima instável, sendo facilmente desnaturada por pH, temperaturas elevadas e em solução aquosa (BANKAR et al., 2009). Suas principais aplicações comerciais são a remoção de glicose em produtos como o ovo em pó e a desoxigenação de produtos como suco de frutas, bebidas engarrafadas e maionese, melhorando a cor, o aroma e a vida útil destes alimentos (BANKAR et al., 2009). Além da aplicação em alimentos, a utilização da glicose oxidase como biosensor para quantificação de glicose em sangue, urina, bebidas e para controle em processos fermentativos está crescendo (RAUF et al., 2006; BANKAR et al., 2009).

A β-galactosidase ou lactase (β-D-galactosideo galactohidrolase, EC 3.2.1.23) hidrolisa as ligações β-D-galactosídicas (WHITAKER, 1994), catalisando a conversão de lactose em glicose e galactose (KATROLIA et al., 2011; JURADO et al., 2002). A β-galactosidase é formada por uma estrutura assimétrica composta de quatro subunidades ligadas entre si, sendo considerada um dímero de dois dímeros. Cada subunidade contém 1024 resíduos de aminoácidos e massa molecular de 119 kDa

(PEREIRA-RODRÍGUEZ et al., 2011). Suas principais aplicações são a hidrólise de lactose, visando aumento de doçura e da solubilidade de produtos lácteos e principalmente a eliminação do fator de intolerância à lactose de alimentos a base de leite, observada principalmente por adultos, que perdem a habilidade de metabolizar este açúcar (WHITAKER, 1994; MAHONEY, 2003). Além disso, uma aplicação recente é a produção de galacto-oligossacarídeos, que tem função prebiótica (PEREIRA-RODRÍGUEZ et al., 2011).

As proteases (EC 3.4.x.x) hidrolisam as proteínas em peptídeos e aminoácidos (SUMANTHA, LARROCHE, PANDEY 2006; MERHEB et al., 2007). Elas fazem parte de um grupo complexo de enzimas que se subdividem em função da especificidade para o substrato, forma do sítio ativo, mecanismo catalítico e pH e temperaturas ótimas de atividade e de estabilidade (SUMANTHA, LARROCHE, PANDEY, 2006). Assim, são subclassificadas em aspártico-proteases, serina-proteases, cisteína-protease e metalo-protease (HARTLEY, 1960). As serina-proteases (EC 3.4.21.62) são apresentam alta concentração de alanina, valina e leucina. Elas são produzidas por micro-organismos do gênero *Bacillus* e constituem o grupo de enzimas de maior importância comercial, representando 35% do mercado de enzimas (ÇALIK et al., 2002). São utilizadas no processo produtivo de detergentes, bebidas, produtos de laticínios, amaciamento de carnes, (MERHEB et al., 2007), modificações de soja para uso em aromas, alimentação animal (SCHALLMEY, SINGH, WARD, 2004), em detergentes e para síntese proteica (SUMANTHA, LARROCHE, PANDEY, 2006).

Industrialmente, as reações enzimáticas podem ser utilizadas para substituir algumas reações químicas e apresentam as vantagens de serem específicas, não apresentarem toxicidade, terem alta eficiência catalítica e baixo custo energético, utilizarem condições mais brandas (pH, temperatura) e serem de mais fácil controle (KRAJEWSKA, 2004). Por outro lado, a aplicação de enzimas em grandes quantidades apresenta dois principais obstáculos, que são o custo das enzimas e a baixa estabilidade em condições de processos que utilizam altas temperaturas, altas concentrações salinas, valores de pH diversos (EISENMENGER, REYES-DE-CORCUERA, 2009b; KRAJEWSKA, 2004; IYER, ANANTHANARAYAN, 2008),

presença de surfactante (IYER, ANANTHANARAYAN, 2008) e alta concentração de substrato (BARTON, BULLOCK, WEIR, 1996).

Algumas tecnologias foram desenvolvidas para minimizar essas limitações. Uma produção de enzima mais eficiente e mais barata, utilizando como meio de fermentação subprodutos da indústria de alimentos é uma das formas mais estudadas para viabilização do custo de produção das enzimas (BEROVIC, OSTROVERSNIK, 1997; KONA, QUERESHI, PAI, 2001). Outros métodos utilizados para aumento de atividade catalítica e da estabilidade enzimática são a realização de operações do processo produtivo em meio não aquoso (KHMELNITSKY et al., 1988; LEVITSKY, LOZANO, IBORRA, 2000), a utilização de engenharia genética (HAKI, RAKSHIT, 2003; IYER, ANANTHANARAYAN, 2008) e de proteínas (IYER, ANANTHANARAYAN, 2008) e a imobilização de enzimas (KRAJEWSKA, 2004; MATEO et al., 2007; EISENMENGER e REYES-DE-CORCUERA, 2009 a,b)

Além desses métodos, que já foram extensivamente avaliados, a aplicação de tecnologias não convencionais, como ultrassom, micro-ondas e alta pressão, começou a ser estudada para ativação e estabilização enzimática, e aumento de ação no meio reativo (CANO, HERNÁNDEZ, ANCOS, 1997; BARTON, BULLOCK, WEIR, 1996; REJASSE et al., 2007).

O ultrassom, utilizado durante a ação das enzimas sobre o substrato, resulta em aumento de atividade por melhorar a difusividade e transferência de massa de produtos e substratos (LEE et al., 2008; JIAN, WENYI, WUYONG, 2008). Isto torna os processos mais efetivos principalmente por aumentar o contato entre substrato e enzima (JIAN, WENYI, WUYONG, 2008), especialmente quando se trata de enzimas imobilizadas (MASON, PANIWNYK, LORIMER, 1996) ou existe algum tipo de inibição (BARTON, BULLOCK, WEIR, 1996). Além disso, o processo também pode promover alterações no substrato, como quebra de regiões helicoidais, favorecendo o acesso da enzima (JIAN, WENYI, WUYONG, 2008).

O processamento por micro-ondas foi estudado por alguns autores e a resposta obtida pode ser relacionada com o meio de reação, sendo que, em meio aquoso não há alteração da atividade enzimática, enquanto que, em meio não aquoso, há aumento de atividade, estabilidade e/ou seletividade enzimática após

aplicação de micro-ondas de baixa energia (ROY, GUPTA, 2003). Isso pode ser explicado pela transferência direta de energia do campo magnético para as frações polares das enzimas, aumentando a flexibilidade das mesmas, a sua reatividade (REJASSE et al., 2007) e também as colisões entre enzima e substrato (YADAV, LATHI, 2005). Resultados obtidos para enzimas previamente tratadas por micro-ondas também indicam aumento de atividade (REJASSE et al., 2007).

O tratamento por alta pressão hidrostática (APH) foi capaz de promover a ativação (MOZHAEV et al., 1996; SILA et al., 2007; EISNMENGER, REYES-DE-CORCUERA, 2009a, b; CAO et al., 2011) e estabilização (MOZHAEV et al., 1996; EISNMENGER, REYES-DE-CORCUERA, 2009a, b) de enzimas, pela aplicação de baixas pressões (até 400 MPa) e temperaturas moderadas (KUDRYASHOVA, MOZHAEV, BALNY, 1998; KNNOR, 1999; SILA et al., 2007). Além destas variáveis, o efeito nas enzimas também depende do solvente e do substrato utilizado (EISNMENGER, REYES-DE-CORCUERA, 2009a). O aumento de atividade foi relatado tanto em enzimas previamente processadas por APH (CANO, HERNÁNDEZ, ANCOS, 1997; KATSAROS, GIANNOGLOU, TAOUKIS, 2009; CAO et al., 2011) como nas reações enzimáticas conduzidas a alta pressão (MOZHAEV et al., 1996; KUDRYASHOVA, MOZHAEV, BALNY, 1998; SILA et al., 2007; EISNMENGER, REYES-DE-CORCUERA, 2009b).

Eisenmenger e Reyes-de-Corcuera (2009) observaram aumento da atividade e da estabilidade térmica de lipase imobilizada em tratamentos de até 350 MPa. Katsaros, Giannoglou, Taoukis (2009) avaliaram a ativação de 5 aminopeptidases e obtiveram aumento médio de atividade de 2-3 vezes após o tratamento a 200 MPa por 20 minutos para 4 enzimas. Mozhaev et al. (1996) observaram aumento de aproximadamente 30 vezes na atividade α-quimotripsina após tratamento a 360 MPa/ 50ºC e perda de atividade em pressões superiores devido a sua desnaturação. Sila et al. (2007) observaram aumento de atividade de PME de 10-20 vezes após processamento a 300-400 MPa/ 50ºC. Kudryashova, Mozhaev e Balny (1998) observaram atividade de termolisina 18 e 23 vezes maior após os processamentos a 40ºC/ 250 MPa e 60ºC/ 150MPa, respectivamente e, perda de atividade em temperaturas superiores a 80ºC, pela inativação da enzima. Cao et al. (2011)

observaram aumento de 17% em β-glucosidase presente naturalmente em polpa de morango após o tratamento a 400 MPa/ 25 minutos.

Segundo Knnor (1999) a ativação de enzimas pela alta pressão é observada apenas para enzimas monoméricas, entretanto, segundo Eisnmenger e Reyes-De-Corcuera (2009a), a ativação por APH já foi relatada para pelo menos 15 enzimas diméricas ou tetraméricas. Para cada enzima, há um limite de pressão a ser aplicada, a partir do qual se observa perda de atividade devido à desnaturação (KUDRYASHOVA, MOZHAEV, BALNY, 1998; SILA et al., 2007; EISENMENGER, REYES-DE-CORCUERA, 2009a). O processo de pressurização, seguindo o princípio de *Le Chatelier*, induz a redução de volume molecular (KNNOR, 1999), acelerando a ocorrência de reações favorecidas nessas condições (MOZHAEV et al., 1996). Em termos moleculares, a APH pode induzir a ativação pelo aumento da flexibilidade conformacional das enzimas gerado pela hidratação dos seus grupos carregados (EISENMENGER, REYES-DE-CORCUERA, 2009a, b), aumento das interações físicas da molécula com o substrato (EISENMENGER, REYES-DE-CORCUERA, 2009a) e aumento da concentração de grupos polares e carregados no complexo de Michaelis e no estado de transição (KUDRYASHOVA, MOZHAEV, BALNY, 1998), com consequente aumento na taxa das reações. Além disso, a pressurização também pode provocar a alteração do fator limitante da reação (concentração mínima de substrato ou produto para a ocorrência da reação) ou alteração no meio de reação/substrato, aumentando a velocidade da reação (EISENMENGER, REYES-DE-CORCUERA, 2009a).

A estabilização das enzimas pela pressão pode ser explicada pela interação intramolecular, hidratação de grupos carregados, quebra de água ligada e estabilização das pontes de hidrogênio (EISENMENGER, REYES-DE-CORCUERA, 2009 a, b), sendo a hidratação de grupos carregados e não polares o principal fator capaz de fortalecer a hidratação das proteínas, prevenindo a desnaturação térmica.

1.1.2. Homogeneização à alta pressão

A homogeneização à alta pressão (HAP), também chamada de homogeneização a ultra-alta pressão ou alta pressão dinâmica é um processo físico não térmico utilizado para o processamento de alimentos visando a sua conservação (DIELS; MICHIELS, 2006; CAMPOS, CRISTIANINI, 2007; TRIBST et al., 2009, WELTI-CHANES, OCHOA-VELASCO, GUERRERO-BÉLTRAN, 2009; TRIBST et al., 2011; FRANCHI, TRIBST, CRISTIANINI, 2011a) e também o aumento de sua estabilidade física, através da diminuição de separação de fases e aumento da sua consistência (FLOURY et al., 2002; FLOURY et al., 2004; MASSON et al., 2011, AUGUSTO, IBARZ, CRISTIANINI, 2012a, b).

Este processo é descrito para aplicação em alimentos fluidos (TORREZAN, 2003) e surgiu a partir dos processos comuns para homogeneização de produtos lácteos e emulsões, tendo, o mesmo princípio de operação (DIELS, MICHIELS, 2006), porém utilizando-se pressões da ordem de 10 a 15 vezes superiores às habitualmente aplicadas, ou seja, pressões de até 350 MPa (3500 bar). No equipamento, o fluido é impelido a passar por uma válvula de homogeneização à altas pressões (TORREZAN, 2003). A passagem pelo orifício estreito da válvula (da ordem de micrometros) e a descompressão abrupta do fluido geram um aumento da sua velocidade (entre 150 e 300 $m.s^{-1}$ – FLOURY et al., 2004; PINHO et al., 2011) e também aumento de temperatura (em torno de 2 a 2,5 ºC a cada incremento de pressão de 10 MPa – DIELS, MICHIELS, 2006) causado pelo atrito intenso na região da válvula de homogeneização (FLOURY et al., 2004). A pressão de operação é controlada pela distância entre a válvula de homogeneização e seu cabeçote (DIELS, MICHIELS, 2006). O apêndice I traz uma ilustração esquemática do equipamento de homogeneização à alta pressão utilizado no presente projeto.

Apesar de não estar plenamente elucidado, o mecanismo de inativação microbiana por HAP é vinculado ao rompimento celular (DIELS, TAEYE, MICHIELS, 2005). Este efeito é causado pelo atrito, cisalhamento, fricção e cavitação que ocorrem no momento em que o fluido passa pela válvula de homogeneização (MIDDELBERG, 1995; KLEINIG, MIDDELBERG, 1998), devido ao espaço restrito

para a passagem do fluido, às velocidades atingidas ou à queda brusca de pressão. Acredita-se que todos esses mecanismos sejam válidos, mas não há consenso sobre qual deles é o mais relevante para o processo (INNINGS, TRÄGARDH, 2007).

A HAP foi descrita como capaz de inativar células vegetativas de bactérias (GUERZONI et al., 1999; WUYTACH, DIELS, MICHIELS, 2002; CAMPOS, CRISTIANINI, 2007; TAHIRI et al., 2006; BRIÑEZ et al., 2007; TRIBST, FRANCHI, CRISTIANINI, 2008; FRANCHI; TRIBST, CRISTIANINI, 2011b; FRANCHI; TRIBST, CRISTIANINI, 2012), leveduras (FANTIN et al., 1996; GECIOVA, BURY, JELEN, 2002; TAHIRI et al., 2006; FRANCHI, TRIBST, CRISTIANINI, 2011b) e bolores (TAHIRI et al., 2006; TRIBST et al., 2009; TRIBST et al., 2011). Os primeiros trabalhos publicados sobre o processo indicaram que o mesmo não era capaz de promover efeito subletal em micro-organismos (WUYTACH, DIELS, MICHIELS, 2002; DIELS, TAEYE e MICHIELS, 2005; BRIÑEZ et al. 2007), entretanto, resultados recentes demonstraram que a HAP pode ter ação sinérgica com tratamento térmico brando para inativação de conídios de *Aspergillus niger* (TRIBST et al., 2009) e de esporos de *Bacillus cereus* e *Bacillus subtilis* (CHAVES-LÓPEZ et al., 2009).

Adicionalmente à inativação microbiana, o efeito da homogeneização sobre as macromoléculas presentes em alimentos tem sido estudado, destacando-se os efeitos em proteínas (SUBIRADE et al., 1998; BOUAUINA et al., 2006; GÁRCIA-JULIÁ et al., 2008, KEERATI-U-RAI, CORREDIG, 2009; LUO et al., 2010; DONG et al., 2011; YUAN et al., 2012), carboidratos (LAGOUEYETE, PAQUIN, 1998; FLOURY et al., 2002; LACROIX, FLISS, MAKHLOUF, 2005; MODIG et al., 2006; KIVELÄ et al., 2010; VILLAY et al., 2012), e lipídeos (KHEADR et al. 2002; KIELCZEWSKA et al. 2003; HAYES e KELLY, 2003; SERRA et al., 2007).·.

Muitos estudos avaliaram o efeito da homogeneização em diferentes tipos de proteínas, sendo observado que o processo foi capaz de alterar a conformação da proteína em alguns casos (GÁRCIA-JULIÁ et al., 2008; LUO et al., 2010; DONG et al., 2011; YUAN et al., 2012), enquanto em outros nenhuma alteração foi observada (BOUAUINA et al., 2006). Os diferentes efeitos podem ser relacionados com o tipo de proteína avaliada, condições de processo e pressões estudadas.

A homogeneização à alta pressão é capaz de fornecer energia suficiente para a quebra das estruturas quaternárias e terciária da maioria das proteínas globulares (SUBIRADE et al., 1998), o que pode levar ao rearranjo e a formação de novos agregados proteicos (KEERATI-U-RAI, CORREDIG, 2009).

A desnaturação e dissociação são efeitos relatados pelo processo de HAP em proteína (DONG et al., 2011), aumentando a área de exposição (DONG et al., 2011) e a quebra da proteína (LUO et al., 2010), com consequente redução da massa molecular (DONG et al., 2011), aumento do poder redutor e eliminação de radicais hidroxila, que são os grupos de maior potencial ativo das proteínas (DONG et al., 2011).

Além disso, para algumas proteínas foi observado a formação de novas estruturas secundárias (LUO et al., 2010) e aumento das interações hidrofóbicas (GÁRCIA-JULIÁ et al., 2008; LUO et al., 2010; YUAN et al., 2012), resultando em formação de agregados proteicos (GÁRCIA-JULIÁ et al., 2008; KEERATI-U-RAI, CORREDIG, 2009; LUO et al., 2010; YUAN et al., 2012).

Para estes agregados foi observado uma solubilidade superior a da proteína nativa (LUO et al., 2010; YUAN et al., 2012), o que foi atribuído à formação de uma fina camada de agregados solúveis adsorvida na proteína (LUO et al., 2010) ou ao aumento da flexibilidade da estrutura proteica.

Assim, a avaliação dos resultados da HAP sobre proteínas mostra que o processo tem muitos efeitos sobre as estruturas proteicas, com consequentes alterações em suas funcionalidades.

Em polissacarídeos, a HAP causa redução do tamanho de partícula (FLOURY et al., 2002; LACROIX, FLISS, MAKHLOUF, 2005; MODIG et al., 2006; KIVELÄ et al., 2010; VILLAY et al., 2012), redução da massa molecular devido a quebra de ligações covalentes (LAGOUEYETE, PAQUIN, 1998; FLOURY et al., 2002; MODIG et al., 2006; VILLAY et al., 2012) e mudanças conformacionais (FLOURY et al., 2002; MODIG et al., 2006; KIVELÄ et al., 2010; VILLAY et al., 2012). Estas mudanças têm como principal efeito a redução na consistência/ viscosidade de soluções e produtos contendo polissacarídeos (AUGUSTO, IBARZ, CRISTIANNINI, 2012; HARTE, VENEGAS, 2010; LANDER et al., 2000; LAGOUEYE, PAQUIM, 1998).

Diferentes níveis de modificações em polissacarídeos foram observados em estudos distintos. Os resultados foram normalmente dependentes do nível de pressão, número de tratamentos sucessivos no equipamento e do tipo de polissacarídeo avaliado. Foi observado que a estrutura do polissacarídeo e sua conformação (linear/ramificada) têm maior influência sobre os efeitos da HAP do que a carga do polímero ou sua massa molar (VILLAY et al., 2012).

O processo de HAP promove uma etapa de transição conformacional (abertura da molécula) seguida da degradação do polímero, que é causada pelo estresse mecânico (LAGOUEYE, PAQUIM, 1998; VILLAY et al., 2012). Segundo Lander et al. (2000), o cisalhamento durante a homogeneização é o principal mecanismo responsável pela quebra do polissacarídeo, enquanto que os efeitos da cavitação podem ser desconsiderados.

A avaliação do efeito de múltiplas passagens em polissacarídeos demonstrou que o processo causou uma despolimerização, quebra de cadeia e redução do tamanho molecular de forma contínua, porém com o maior impacto na primeira passagem (LAGOUEYE, PAQUIM, 1998; KIVELÄ et al., 2010; VILLAY et al., 2012). Este fato está relacionado com o mínimo tamanho molecular obtido após cada pressão, que é diferente em função do nível de pressão aplicada, mas pouco alterada pelo número de vezes que a solução de polissacarídeo é submetida ao processo de HAP (LAGOUEYE, PAQUIM, 1998; VILLAY et al., 2012). Isto pode ser explicado considerando-se que, a cada passagem pelo homogeneizador a amostra é submetida à mesma magnitude de tensão, e, consequentemente à mesma energia mecânica. A quebra molecular está diretamente ligada com a ruptura de ligações que tem menor nível energético do que a quantidade de energia fornecida no processo, consequentemente, a degradação do polissacarídeo tem um comportamento assintótico após múltiplas passagens no homogeneizador (HARTE, VENEGAS, 2010; LANDER et al., 2000; LAGOUEYE, PAQUIM, 1998).

Por outro lado, segundo Lagoueyete e Paquin (1998), após a abertura da cadeia polimérica, ela pode se tornar mais susceptível às degradações induzidas pelo processo, uma vez que a HAP deixa exposto um grande número de agrupamentos que se tornam mais sensíveis a homogeneização subsequente.

O efeito da homogeneização sobre lipídeos foi medido principalmente em gordura do leite, visto que o processo é vastamente estudado para aplicação em produtos lácteos. Segundo Kielczewska et al. (2003) e Kheadr et al. (2002), a HAP melhora a dispersão e reduz os glóbulos de gordura. A temperatura da matriz gordurosa influencia na redução dos glóbulos, pois o processo é mais efetivo quando toda a gordura está líquida durante a descompressão (THIEBAUD et al., 2003), ou seja, quando a temperatura do leite é superior a 40ºC.

A redução dos glóbulos de gordura é função do nível de pressão aplicada no processo de HAP, porém utilizando-se pressões entre 50 e 300 MPa observa-se reduções maiores do que as obtidas pelo processo de homogeneização comum (SANDRA, DALGLEISH, 2005). Em pressões de homogeneização superiores a 300 MPa (THIEBAUD et al., 2003; SERRA et al., 2007), por outro lado, um efeito contrário é observado, visto que a intensa redução dos glóbulos modifica sua carga elétrica e favorece a coalescência (SERRA et al., 2007; THIEBAUD et al., 2003). Este fenômeno pode ser minimizado pela utilização de dodecil sulfato de sódio (THIEBAUD et al., 2003) e pela realização da homogeneização em 2 estágios (HAYES, KELLY, 2003).

A avaliação de gorduras de leites de vaca, cabra e ovelha demonstrou que a homogeneização até 350MPa não altera o perfil de ácidos graxos das amostras ou os isômeros de ácidos linoleicos conjugados (RODRÍGUEZ-ALCALÁ, HARTE, FONTECHA, 2009), indicando que, apesar da homogeneização ser capaz de quebrar os glóbulos de gordura, o processo não altera a gordura quimicamente.

O efeito destas modificações em carboidratos, proteínas e lipídeos abriu uma nova frente de aplicação do processo de homogeneização, visando a alteração da estrutura de alimentos, entre os quais se destaca a obtenção de produtos lácteos fermentados a partir de leite homogeneizado (GUERZONI et al. 1999; LANCIOTTI et al. 2004; BOUAUINA et al. 2006; PATRIGNANI et al. 2007; SERRA et al. 2009), obtendo-se as seguintes melhorias:

a. O aumento de atividade proteolítica e lipolítica durante a maturação de queijos, provocada pelo aumento da área de exposição de lipídeos e desnaturação proteica parcial (GUERZONI et al., 1999);

b. Maximização do crescimento de culturas *starters* durante a fermentação e redução da perda de viabilidade das mesmas durante a estocagem refrigerada, devido à proteção celular pela proteína homogeneizada (LANCIOTTI et al. 2004; PATRIGNANI et al., 2007);
c. Aumento dos peptídeos hidrofóbicos que tem um potencial bioativo durante a fermentação (SERRA et al., 2009);
d. Redução da sinérese e aumento da firmeza do leite fermentado, em função da rede proteica formada após a homogeneização, com agregação das proteínas do soro à caseína (PATRIGNANI et al., 2007; SERRA et al., 2009);
e. Melhoria do perfil aromático dos produtos fermentados, em função dos aminoácidos liberados durante a fermentação da proteína modificada pela homogeneização (PATRIGNANI et al., 2007).

1.1.3. Homogeneização à alta pressão e o efeito sobre enzimas

Estudos demonstram que a HAP afeta a estabilidade e a atividade de enzimas, bem como de outras macromoléculas. Este é, entretanto, um foco relativamente recente quando comparado à avaliação do processo frente a proteínas, lipídeos e carboidratos.

A maioria dos estudos de aplicação da HAP em enzimas foi realizada com o propósito de inativar enzimas indesejáveis em alimentos processados, como é o caso da peroxidase em água de coco (DOSUALDO, 2007), polifenoloxidase em água de coco (DOSUALDO, 2007), peras (LIU et al., 2009a) e cogumelos (LIU et al., 2009b) e pectina metilesterase em sucos de laranja (LACROIX, FLISS, MAKHLOUF, 2005; WELTI-CHANES, OCHOA-VELASCO, GUERRERO-BÉLTRAN, 2009; VELÁZQUEZ-ESTRADA et al., 2012) e banana (CALLIGARIS et al., 2012). Alguns trabalhos também avaliaram o efeito do processo em enzimas com função antimicrobiana, como é o caso da lisozima (TRIBST, FRANCHI, CRISTIANINI, 2008; FRANCHI, TRIBST, CRISTIANINI, 2011b), lactoperoxidase (VANNINI et al., 2004; IUCCI et al., 2007) e lactoferrina (IUCCI et al., 2007). Outros avaliaram ainda a atividade de enzimas nativas de alimentos, como a plasmina (PINHO, 2006),

resultantes do crescimento microbiano em leite (GUERZONI et al., 1999; LANCIOTTI et al., 2004; PAREDA et al., 2008; VANNINI et al., 2008).

Estes trabalhos relataram que o processo foi capaz de ativar, inativar ou não alterar a atividade das enzimas estudadas. A ativação enzimática foi observada quando aplicadas baixas pressões, com aumento de 80% para polifenoloxidase em pera após 3 tratamentos consecutivos a 140 MPa ou a 2 tratamentos consecutivos a 160 MPa, sendo que o aumento de temperatura de entrada entre 25 e 45ºC resultou em aumento de atividade em 30% para amostras processadas a 140 MPa (LIU et al., 2009a). Liu et al (2009b) observou aumento de 10% na atividade de polifenoloxidase extraída de cogumelos e que este aumento teve pequenos acréscimos com a realização de processos sequenciais no equipamento. Dosualdo (2007) observou um aumento de atividade em torno de 50% para polifenoloxidase e de 11% para peroxidase após a HAP de água de coco a 44 MPa em valores de pH de 4,7 e 5,8, respectivamente. Outros autores, entretanto, relataram apenas redução da atividade enzimática de pectina-metilesterase após a HAP de suco de laranja e banana (LACROIX, FLISS, MAKHLOUF, 2005; WELTI-CHANES, OCHOA-VELASCO, GUERRERO-BÉLTRAN, 2009; VELÁZQUEZ-ESTRADA et al., 2012; CALLIGARIS et al., 2012)

Os resultados obtidos para o efeito da homogeneização sobre enzimas com características antimicrobianas indicaram aumento da atividade antimicrobiana de lisozima e lactoperoxidase a 75 MPa (VANNINI et al., 2004) ou 100 MPa (IUCCI et al., 2007). Tribst, Franchi e Cristianini (2008) e Franchi, Tribst e Cristianini (2011b), por outro lado, observaram perda de atividade de muramidase para lisozima tratada em pressões maiores que 250 MPa, porém, sem perda de atividade antimicrobiana, indicando uma possível modificação dos sítios ativos da molécula. Essa diferença dos resultados obtidos pode ser função dos diferentes micro-organismos alvos utilizados em cada teste.

Trabalhos que avaliaram as características de queijos produzidos com leite processado por HAP 100 MPa indicaram um aumento de atividade de enzimas proteolíticas (excluindo-se a plasmina/plasminogênio, que é inativada com a HAP) e lipolíticas presentes no leite ou oriundas do metabolismo de microrganismos

contaminantes (GUERZONI et al., 1999; HAYES e KELLY, 2003; LANCIOTTI et al., 2004; PINHO, 2006; PAREDA et al., 2008; VANNINI et al., 2008), melhorando o sabor, a textura (VANNINI et al., 2008), a cor e o aroma (LANCIOTTI et al., 2006) e acelerando o processo de cura dos queijos (LANCIOTTI et al., 2006).

Trabalhos mais recentes avaliaram os efeitos da HAP em soluções de enzimas para determinar se o processo é capaz de aumentar a estabilidade ou a atividade de algumas enzimas de interesse comercial (LIU et al., 2010; TRIBST, CRISTIANINI, 2012a – *veja capitulo 3 para maiores detalhes*; TRIBST, AUGUSTO, CRISTIANINI, 2012- *veja capítulo 4 para maiores detalhes;* TRIBST, CRISTIANINI, 2012b – *veja capitulo 5 para maiores detalhes*; TRIBST, AUGUSTO, CRISTIANINI, *in press- veja capítulo 6 para maiores detalhes*; TRIBST, AUGUSTO, CRISTIANINI, *in press- veja capítulo 7 para maiores detalhes*). Liu et al. (2010) observou que o processo, apesar de não promover a ativação de enzimas, tornou-as mais estáveis durante o aquecimento, com aumento de 10% na atividade residual de tripsina após a HAP a 80 MPa e também com aumento de estabilidade ao pH. Tribst e Cristianini (2012a) avaliaram o efeito da HAP até 150 MPa e não observaram alterações na atividade e na estabilidade de α-amilase fúngica a diferentes temperaturas, presença ou ausência de cálcio e durante o armazenamento. Já resultados obtidos por Tribst, Augusto e Cristianini (2012) demonstraram que HAP a 200 MPa alterou a temperatura ótima de atividade de uma protease neutra de *Bacillus subtilis* de 55ºC para 20ºC, com um aumento de cerca de 30% na atividade a 20ºC comparado com a enzima nativa. Resultados obtidos para amiloglicosidase (TRIBST, CRISTIANINI, 2012b) e glicose oxidase (TRIBST, CRISTIANINI, *in press*) indicaram um aumento de atividade em temperaturas acima da ótima e os resultados obtidos para β-galactosidase demostraram que a enzima ficou estável apenas quando utilizadas condições de atividade ótima (TRIBST, AUGUSTO, CRISTIANINI, *in press*).

Assim, observa-se que o efeito do processo de homogeneização sobre a enzima tem ação diferente em função do tipo de enzima e também do nível de pressão aplicada, mas é possível obter, pelo menos para algumas enzimas, ganhos de atividade e ou estabilidade.

O aumento de atividade é normalmente atribuído às alterações causadas pela HAP nas estruturas quaternária, terciária e secundária das enzimas (LACROIX, FLISS, MAKHLOUF, 2005; LIU et al., 2009b; LIU et al., 2010). Estas alterações envolvem:

1. Aumento na exposição dos grupos sulfidrilas superficiais e redução do número total de grupos SH disponíveis, indicando que o processo causa desnaturação e desdobramento molecular ao mesmo tempo em que favorece a formação de pontes de sulfeto (LIU et al., 2009b, LIU et al., 2010),
2. Aumento da exposição dos resíduos de tirosina e triptofano (LIU et al., 2009b, LIU et al., 2010) e alterações da configuração da vizinhança destes aminoácidos (LIU et al., 2009b),
3. Modificação da exposição de grupos hidrofóbicos (LIU et al., 2009b, LIU et al., 2010),
4. Alterações da exposição dos sítios ativos das enzimas (VANNINI et al., 2004; LANCIOTTI et al., 2007; IUCCI et al., 2007),
5. Redução das pontes de hidrogênio inter e intra moleculares (LIU et al., 2010),
6. Mudança nos percentuais de estruturas secundárias (α-hélice e β-pregueada) após a homogeneização (LIU et al., 2009b).

Não foram observados, por outro lado, alterações nas massas moleculares das enzimas, indicando não haver hidrólise da cadeia peptídica nas condições estudadas (LIU et al., 2010). Segundo Vannini et al. (2004), Iucci et al. (2007) e Lanciotti et al. (2007), durante a pressurização há rompimento das estruturas tridimensionais das proteínas, permitindo que os grupos movam-se livremente, independentemente de sua configuração original e, durante a descompressão instantânea, há rearranjo molecular, mas os grupamentos não retornam às suas configurações originais. Lanciotti et al. (2006) sugerem que o aumento de atividade proteolítica em queijos produzidos com leite tratado por HAP seja resultado de uma combinação de pressão e aumento de temperatura, aproximando-se da temperatura ótima de atividade das enzimas proteolíticas. Liu et al. (2009a) e Dosualdo (2007),

por sua vez, sugerem que, além de fatores citados acima, possivelmente há a ativação de isoenzimas ou regeneração da enzima pela HAP.

Considerando-se que a homogeneização à alta pressão é um processo não térmico com potencial aplicação para a alteração da conformação de enzimas visando a melhoria do desempenho das mesmas e que o aumento de estabilidade e atividade são dois fatores cruciais para a ampliação do mercado de aplicações de enzimas, foram estabelecidos os objetivos desta pesquisa.

1.2. Objetivos

O objetivo geral do presente trabalho foi avaliar o efeito da homogeneização à alta pressão em enzimas comerciais. Para uma avaliação global deste objetivo, o mesmo foi subdividido em alguns objetivos específicos:

1. Estabelecer métodos analíticos para a avaliação da atividade de α-amilase de *A. niger*, β-galactosidase de *K. lactis*, amiloglicosidase de *A. niger*, glicose-oxidase de *A. niger* e protease neutra de *B. subtilis* (Capítulo 2).
2. Avaliar o efeito da HAP na atividade e na estabilidade de α-amilase (Capítulo 3), protease neutra (Capítulo 4), amiloglicosidase (Capítulo 5), β-galactosidase (Capítulo 6) e glicose oxidase (Capítulo 7).
3. Avaliar o efeito de múltiplas passagens de soluções de amiloglicosidase, glicose-oxidase e protease no homogeneizador sobre a atividade das enzimas em diferentes temperaturas (Capítulo 8).

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Capítulo 2. Ensaios Preliminares

Resumo

Enzimas são proteínas com funções catalíticas. A atividade das mesmas é função do meio onde estão atuando, da temperatura de ação e pode ser afetada pela concentração de enzima e substrato. Assim é de grande importância a escolha dos parâmetros utilizados durante a realização de uma reação enzimática na qual se tem por objetivo determinar a atividade da enzima. Muitos métodos de determinação de atividade enzimática utilizam reações com substâncias que formam compostos coloridos, cuja concentração é determinada através da leitura de absorbância em espectrôfotometro. Para quantificação dos resultados é necessária a obtenção de curvas padrões nas diferentes condições avaliadas (temperatura e pH), de forma que as mesmas sejam compatíveis com as leituras das amostras após a atividade enzimática. Desta forma o objetivo deste capítulo foi estabelecer as metodologias de determinação de atividade enzimática de protease neutra, α-amilase, glicose oxidase, amiloglicosidase e β-galactosidase e obter as curvas padrões de maltose pelo método açúcares redutores utilizando ácido dinitrosalicilico (Ácido DNS) e glicose, pelos métodos de açúcares redutores utilizando ácido DNS e kit enzimático para determinação de glicose.

Palavras-Chave: Enzimas, metodologias para determinação de atividade enzimática, curvas padrão.

Abstract

Enzymes are proteins with catalyst function. The enzymes catalytic performance is dependent on their environment, concentration of enzymes and substrates and temperature and time of enzymatic reaction. Therefore, the enzymatic reaction parameters chosen are highly important when the enzymes activities need to be evaluated. Several methods of enzymatic activity quantification are carried out applying substances that react with the product of the enzyme reaction, forming colored compounds. Their concentrations are determined through absorbance in spectrophotometer. For quantify these results, it is required to obtain previously the standard curves at each evaluated condition (temperature and pH), being the samples of the standard curve similar to the samples obtained after enzyme activity. Therefore the aim of this chapter were: (i) establish the methodologies to evaluate the activity of neutral protease, glucose oxidase, amyloglucosidase, α-amylase and β-galactosidase and (ii) obtain the standard curves of maltose and glucose through reducing sugar methodology applying di-nitrosalicilic acid (DNSA) and glucose through enzymatic kit of glucose assays.

Keywords: Enzymes, methodology of enzyme activity, standard curves.

2.1. Introdução

Enzimas são proteínas normalmente globulares (DOBLE, KRUTHIVENTI, GAIKAR, 2004) com funções catalíticas (OLEMPSKA-BEER et al., 2006). Elas reagem especificamente com um substrato através do mecanismo conhecido como chave-fechadura (WHITAKER, 2002) formando um complexo enzima-substrato através de pontes de hidrogênio e interações de Van der Walls. Com o fim da reação, ocorre a dissociação do complexo, com a liberação do produto e da enzima em sua forma nativa (DOBLE, KRUTHIVENTI, GAIKAR, 2004).

A atividade das enzimas é afetada pela concentração de enzima e de substrato presente no meio reativo, pelo pH do meio de reação, pelo tempo e temperatura de ação e pela presença de inibidores e ativadores (WHITAKER, 1994).

Cada enzima tem uma condição ótima de ação em termos de pH e temperatura. Temperaturas e pH inferiores ou superiores resultam em redução da atividade enzimática pela modificação estrutural da enzima, com redução da exposição ou da ação dos sítios ativos. Além disto, temperaturas muito elevadas ou pH extremos podem promover a desnaturação irreversível das enzimas, dependendo do tempo de exposição (WHITAKER, 1994).

Para as enzimas que seguem a cinética descrita pela equação de Michaelis-Menten, observa-se que a velocidade da reação enzimática aumenta proporcionalmente com a adição de substratos até atingir uma velocidade máxima. Em concentrações de substrato acima desta, a velocidade da reação se mantém constante no valor máximo. Para algumas enzimas que apresentam inibição pelo substrato, o aumento excessivo da concentração do substrato pode resultar em redução de atividade (WHITAKER, 2002).

O tempo de reação das enzimas é outro fator importante na determinação da atividade. A velocidade de conversão tende a ser maior no início da reação enzimática, tanto pela maior concentração de substrato como devido a possível inibição da reação pelo produto (WHITAKER, 1994). Assim, muitos autores utilizam o conceito de v_0 (velocidade inicial) para avaliar a atividade de enzimas. A v_0 é a velocidade da reação enzimática quando menos do que 5% do produto foi formado

(WHITAKER, 2002). Desta forma, a obtenção de produtos não apresenta necessariamente uma relação linear com a concentração de enzima, a concentração de substrato e o tempo de reação enzimática.

Muitas metodologias para avaliação da atividade enzimática, especialmente quando o produto da reação é um açúcar redutor, utilizam reações com substâncias que formam compostos coloridos (RAMI, DAS, SATYANARAYANA 2000; KONA, QUERESHI, PAI, 2001; JURADO et al., 2002; WONG, ROBERTSON, 2003), o que torna mais fácil, simples e rápida a quantificação da atividade das enzimas. Estas reações, entretanto, necessitam da obtenção de curvas padrão de açúcares no mesmo pH da reação enzimática, de forma que a absorbância da amostra possa ser convertida em concentração de açúcares e, com este dado, se determinar a atividade da enzima.

Desta forma, o objetivo deste capítulo foi escolher e otimizar as metodologias de determinação de atividade enzimática de protease neutra, α-amilase, glicose oxidase, amiloglicosidase e β-galactosidase e obter as curvas padrões de maltose e glicose pelo método de ácido dinitrosalicilico (Ácido DNS) e glicose, através de kit enzimático para determinação de glicose.

2.2. Material e Métodos

2.2.1. Enzimas

As enzimas utilizadas no estudo foram: α-amilase de *Aspergillus niger*, β-galactosidase de *Kluyveromyces lactis*, amiloglicosidase de *A. niger*, glicose oxidase de *A. niger* e protease neutra de *B. subitilis.* Elas foram obtidas como doação da empresa Prozyn Biossolution ®.

2.2.2. Atividade de α-amilase

A atividade da α-amilase foi medida utilizando concentrações de 0,025, 0,01, 0,005, 0,001 e 0,0005 g de enzima diluídas em 100 mL de tampão acetato 0,1 M pH 5,8 contendo 10 mM de cloreto de cálcio. A atividade enzimática foi avaliada pelo

método de açúcares redutores determinados pela reação com ácido 3-5 dinitrosalicilico (WONG, ROBERTSON, 2003) com algumas alterações: 750 µL de solução enzimática foram adicionados a 750 µL de solução 1% de amido solúvel. A reação enzimática foi realizada a 45ºC/ 10 minutos (condição ótima segundo fabricante da enzima). Para paralização da reação, foram adicionados 450 µL de 1% solução de ácido DNS seguido de incubação em banho em ebulição por 5 minutos. As amostras foram então adicionadas de 4,05 mL de tampão acetato 0,1 M pH 5,8, totalizando um volume de 6 mL. A absorbância das amostras foi medida a 547 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). A atividade foi calculada utilizando a curva padrão de maltose. Uma unidade de enzima foi definida como a quantidade de enzima que catalisa a liberação de um µmol de grupos redutores (medidos como maltose) a partir de amido solúvel por minuto de reação.

Foram realizados ensaios com amostras controle contendo apenas amido (volume de enzima substituído pela adição de tampão) e apenas enzima (volume de amido substituído pela adição de tampão). Os ensaios foram realizados em triplicata e conduzidos conforme descrito para as amostras nas quais foram medidas a atividade enzimática.

A curva padrão de maltose foi obtida utilizando soluções contendo 2,7, 1,75, 1,0, 0,8, 0,6, 0,4, 0,2, 0,1 e 0,05 mmols de maltose diluída em tampões acetato (pH 4,6 e 5,2) ou fosfato (pH 5,8 e 7,0). 1,5 mL de solução de maltose foi adicionada de 450 µL de solução 1% de ácido DNS e posteriormente incubada em banho em ebulição por 5 minutos. O volume da amostra foi completado para 6 mL e a absorbância das amostras foi medida a 547 nm, similarmente a metodologia descrita para as amostras de enzima. Os ensaios para obtenção da curva padrão de maltose foram realizados em triplicata.

2.2.3. Atividade de glicose oxidase

A atividade da glicose-oxidase (GO) foi medida utilizando concentrações de 0,03, 0,015, 0,005 e 0,002 g de enzima diluídas em 100 mL de tampão acetato 0,1 M

pH 5,0 contendo 0,02 $g.L^{-1}$ de nitrato de sódio. A atividade enzimática foi avaliada pelo método de açúcares redutores determinados pela reação com ácido 3,5 di-nitrosalicilico (KONA, QUERESHI, PAI, 2001) com algumas alterações: 400 µL de solução enzimática foi adicionada a 400 µL de solução de glicose (4 $g.L^{-1}$) e a 1,2 mL de tampão acetato 0,1 M pH 5,0. A reação enzimática foi realizada a 50ºC/ 30 minutos (condição ótima segundo o fabricante para enzima) e depois foram adicionados 1,5 mL de solução de ácido DNS 1% seguido de incubação em banho em ebulição por 5 minutos. As amostras foram então adicionadas de 6,5 mL de tampão acetato 0,1 M pH 5,8, totalizando um volume de 10 mL. A absorbância das amostras foi medida a 547 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). A atividade foi calculada utilizando a curva padrão de glicose. Tubos contendo apenas glicose (volume de enzima substituído pela adição de tampão) e apenas enzima (volume de glicose substituído pela adição de tampão) foram utilizados como controle. Os ensaios controle foram realizados em triplicata e conduzidos conforme descrito para as amostras nas quais foram medidas a atividade enzimática.

A atividade da enzima foi calculada pela diferença de absorbância entre as amostras contendo apenas glicose e aquelas adicionadas de glicose e enzima (na qual parte da glicose foi oxidada pela ação da enzima). Uma unidade de enzima foi definida como a quantidade de enzima que converte 1 µg de glicose por minuto.

A curva padrão de glicose foi obtida utilizando soluções contendo 1,8, 1,4, 1,0, 0,7, 0,5 e 0,2 mmols de glicose diluída em tampões acetato (pH 3,6-5,7) ou fosfato (pH 5,8-6,5). Aliquotas de 2 mL de cada solução de glicose foram adicionados de 1,5 mL de solução 1% de ácido DNS, e posteriormente incubada em banho em ebulição por 5 minutos. O volume da amostra foi completado para 10 mL e a absorbância das amostras foi medida a 547 nm, similarmente a metodologia descrita para as amostras de enzima. Os ensaios de determinação de glicose para obenção da curva padrão foram realizados em triplicata.

2.2.4. Atividade de Amiloglicosidase

A atividade da amiloglicosidase (AMG) foi medida utilizando concentrações de enzima de 0,01, 0,05, 0,01, e 0,005g diluídas em 100 mL de tampão acetato 0,05 M pH 4,3. A atividade enzimática foi avaliada pelo método de determinação de glicose através de kit enzimático de glicose oxidase (RAMI, DAS, SATYANARAYANA, 2000) com algumas alterações: 500 µL de solução enzimática foram adicionados a 4 mL de solução de 0,5% de amido solúvel. A reação foi realizada a 65ºC / 10 minutos (condição ótima segundo o fornecedor da enzima) e depois paralisada pela adição de 3 mL de tampão Tris-HCl 1M pH 7,5. A hidrólise do amido foi quantificada pela liberação de glicose em 0,1 mL de amostra, a qual foi medida utilizando-se 4 mL de um kit enzimático de glicose-oxidase (Laborlab, Brasil). A determinação da glicose pelo kit de glicose oxidase foi realizada a 37ºC/ 10 minutos (FLEMING, PEGLER, 1963). O kit contém as enzimas glicose oxidase (GO) e peroxidase (POD), além do reativo de cor 4-aminofenazona (4AAP), e a reação ocorre conforme descrito pelas *Equações 2.1* e *2.2*, sendo que a 4 antipirilquinona é um composto químico avermelhado cuja concentração é medida através de leitura em espectrofotômetro a 510 nm e tem relação direta com a concentração inicial de glicose no meio reativo.

$$\text{Glicose} + O_2 + H_2O \text{ -----GO---> Ácido Glucônico} + H_2O_2 \qquad (\textit{Equação 2.1})$$

$$2\ H_2O_2 + 4\text{AAP ----POD---> 4-antipirilquinomina} + 4\ H_2O \qquad (\textit{Equação 2.2})$$

A absorbância das amostras foi então medida a 510 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). Uma unidade de atividade da enzima é definida como a quantidade de enzima capaz de liberar um µmol de glicose durante o período de reação, ou seja, 10 minutos.

Tubos contendo apenas amido (volume de enzima substituído pela adição de tampão) e apenas enzima (volume de amido substituído pela adição de tampão) foram utilizados como controle. Os ensaios de quantificação de glicose foram realizados em triplicata e conforme descritos acima.

A curva padrão de glicose foi obtida utilizando soluções contendo 1,25, 2,5, 5, 10, 20, 40, 60, 80 e 100 mmols de glicose diluída em 4,5 mL de água e adicionada em 3 mL de tampão Tris-HCl 1M pH 7,5. Aliquotas de 0,1 mL destas soluções de glicose adicionadas à 4 mL de kit enzimático de glicose oxidase. A reação ocorreu a 37ºC / 10 minutos (FLEMING, PEGLER, 1963). A absorbância das amostras foi então medida a 510 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). Os ensaios de determinação de glicose para obenção da curva padrão foram realizados em triplicata.

2.2.5. Atividade de β-galactosidase

A atividade da β-galactosidase (β-GL) foi medida utilizando concentrações de 0,5 e 1 g de enzima diluídas em 100 mL de tampão fosfato 0,1, M pH 7,0. A atividade enzimática foi avaliada pelo método de determinação de glicose através de kit enzimático de glicose oxidase (JURADO et al., 2002) com algumas alterações: 300 µL de solução de β-galactosidase foram adicionados a 3 mL de solução 2 ou 3% de de lactose. A reação foi realizada a 35ºC por 15 ou 30 minutos e depois paralisada pela imersão dos tubos banho em ebulição por 5 minutos. A hidrólise da lactose pela β-GL foi quantificada pela liberação de glicose na amostra, a qual foi medida utilizando-se 0,1 mL da mistura de reação e 4 mL de reagente enzimático de glicose-oxidase (Laborlab, Brasil). A reação da amostra com o reagente enzimático foi realizada a 37ºC/ 10 minutos (FLEMING, PEGLER, 1963). A absorbância das amostras foi então medida a 510 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). Os ensaios de atividade foram realizados em triplicata. Uma unidade de atividade de β-galactosidase foi definida como a quantidade de enzima capaz de liberar um µmol de glicose por minuto de reação.

A curva padrão de glicose foi obtida utilizando soluções contendo 50, 125, 250, 500, 1000, 1500, 2000 e 2500 $mg.L^{-1}$, preparadas em tampões acetato (pH 4,3-5,7), fosfato (pH 6,4-7,0) ou tris-HCl (7,5-9,0). As misturas de 0,1 mL de solução de glicose foram adicionadas de 4 mL de reagente de glicose oxidase, e a reação

enzimática realizada conforme descrito acima. Os ensaios da curva padrão foram realizados em triplicata.

2.2.6. Protease Neutra

A atividade da protease neutra foi medida utilizando concentrações de 0,25, 0,125, 0,1 e 0,05 g de enzima diluídas em 100 mL de tampão fosfato 0,1 M pH 7,5. A atividade enzimática foi medida pelo método de hidrólise da caseína (MERHEB et al., 2007) com algumas modificações: 200 µL de solução de enzima foi adicionado a 400 µL de tampão fosfato 0,1M pH 7,5 e 400 µL de solução de caseína 0,5% preparada no mesmo tampão. A reação enzimática foi realizada a 55ºC/ 30 minutos (condição ótima segundo o fornecedor da enzima) e, em seguida, foi adicionado 1 mL de solução 10% de ácido tricloroacético (TCA) para paralização da reação. As amostras foram então centrifugadas, para separação das proteínas precipitadas, a 10.000 rpm/ 5 min/ 10ºC e suas absorbâncias foram medidas a 275 nm utilizando um espectrofotômetro UV-visível DU 800 (Beckman Coulter ®, Brea, CA). A amostra controle foi obtida pela adição do TCA nos tubos antes da adição da enzima. Uma unidade de enzima foi definida como a quantidade de enzima necessária para aumentar em 0,1 a absorbância da amostra a 275 nm, nas condições do teste. O valor de ΔAbs_{275nm} foi determinado através da diferença de absorbância entre as amostras e o controle. A atividade enzimática foi calculada a partir da *Equação 2.3*, descrita por Merheb et al, 2007.

$$U/g = \Delta Abs_{275nm} \cdot 10 \cdot \text{fator de diluição} / (0.2) \qquad (\textit{Equação 2.3})$$

2.3. Resultados e Discussões

As curvas padrão de maltose foram obtidas nos pH (4,6, 5,2, 5,8 e 7,0) em que a α-amilase foi avaliada. A *Tabela 2.1* mostra as equações obtidas pelas regressões lineares destas curvas e os seus respectivos valores de R^2. Os resultados mostraram que o pH afetou significativamente a inclinação das curvas padrão, o que era esperado uma vez que o cromóforo do reativo é dependente do pH

e também pode ser afetado pelos sais presentes no meio, em função dos diferentes tampões utilizados. Cada curva padrão foi utilizada para determinação da atividade da α-amilase avaliada nos valores de pH específicos.

Tabela 2.1. Regressões obtidas para as curvas padrão de maltose

pH das amostras	Regressões lineares	R^2
pH 4,6	[maltose $_{(mmol)}$] = 10,63*abs_{547nm} + 0,04	0,99
pH 5,2	[maltose $_{(mmol)}$] = 8,09*abs_{547nm} + 0,01	>0,99
pH 5,8	[maltose $_{(mmol)}$] = 12,23*abs_{547nm} + 0,16	0,98
pH 7,0	[maltose $_{(mmol)}$] = 3,28*abs_{547nm} + 0,10	0,96

As curvas padrão de glicose (determinadas através do método de quantificação de açúcares redutores pela reação com ácido DNS) foram obtidas nos valores de pH (3,6-6,5) em que a GO foi avaliada. A *Tabela 2.2* mostra as equações obtidas pelas regressões lineares destas curvas e os seus respectivos valores de R^2. O pH da solução afetou consideravelmente as leituras de absorbância obtidas e, consequentemente, as inclinações das curvas padrão. Cada curva padrão foi utilizada para determinação da atividade da glicose oxidase nos valores de pH específicos.

Tabela 2.2. Regressões obtidas para as curvas padrão de glicose pelo método de quantificação de açúcares redutores pela reação com ácido DNS

pH das amostras	Regressões lineares	R^2
pH 3,6	[glicose $_{(mg.L^{-1})}$] = 2210,50*abs_{547nm} - 87,85	>0,99
pH 4,3	[glicose $_{(mg.L^{-1})}$] = 2085,90*abs_{547nm} + 0,62	>0,99
pH 5,0	[glicose $_{(mg.L^{-1})}$] = 347,32*abs_{547nm} + 9,61	>0,99
pH 5,7	[glicose $_{(mg.L^{-1})}$] = 309,22*abs_{547nm} + 9,82	>0,99
pH 6,5	[glicose $_{(mg.L^{-1})}$] = 974,42*abs_{547nm} - 0,47	>0,99

Para as análises de β-GL foi necessária a obtenção de curvas padrão de glicose utilizando-se o kit enzimático de glicose oxidase para quantificação de glicose. Para obtenção destas curvas padrão foram utilizadas soluções de glicose

nos pH (4,3-9,0). A *Tabela 2.3* mostra as equações obtidas pelas regressões lineares destas curvas e os seus respectivos valores de R^2. A principal diferença observada entre as curvas foi a inclinação das mesmas.

Tabela 2.3. Regressões das curvas padrão pelo método de quantificação de glicose pela reação com kit enzimático de glicose oxidase

pH das amostras	Regressões lineares	R^2
pH 4,3	[glicose $_{(\mu mol)}$] = 442,84*abs_{510nm} – 20,87	>0,99
pH 5,0	[glicose $_{(\mu mol)}$] = 317,97*abs_{510nm} – 13,38	>0,99
pH 5,7	[glicose $_{(\mu mol)}$] = 290,02*abs_{510nm} – 24,85	>0,99
pH 6,4	[glicose $_{(\mu mol)}$] = 197,16*abs_{510nm} – 5,31	>0,99
pH 7,0	[glicose $_{(\mu mol)}$] = 185,64*abs_{510nm} – 3,66	0,98
pH 7,5	[glicose $_{(\mu mol)}$] = 189,11*abs_{510nm} – 7,41	>0,99
pH 8,0	[[glicose $_{(\mu mol)}$] = 194,19*abs_{510nm} – 9,95	>0,99
pH 8,5	[glicose $_{(\mu mol)}$] = 269,85*abs_{510nm} + 1,36	>0,99
pH 9,0	[[glicose $_{(\mu mol)}$] = 254,45*abs_{510nm} + 1,38	>0,99

Para os ensaios de AMG, a curva padrão foi obtida apenas em tampão Tris-HCl 1 M pH 7,5, uma vez que a alta concentração deste tampão utilizado para paralisar a reação de hidrólise do amido pela AMG fez com que todas as amostras passassem para o pH 7,5 antes da reação com o kit de glicose oxidase, independentemente do pH inicial da mesma. A *Figura 2.1* mostra a regressão obtida para a curva de glicose medida através de kit enzimático de GO.

As curvas padrão mostradas nas *Tabelas 2.1, 2.2* e *2.3* e na *Figura 2.1* ilustram as condições em que as mesmas foram obtidas e como o pH afeta a absorbância dos compostos coloridos formados, seja pela reação dos açúcares com o ácido DNS ou pela reação da glicose com o reagente enzimático de glicose oxidase.

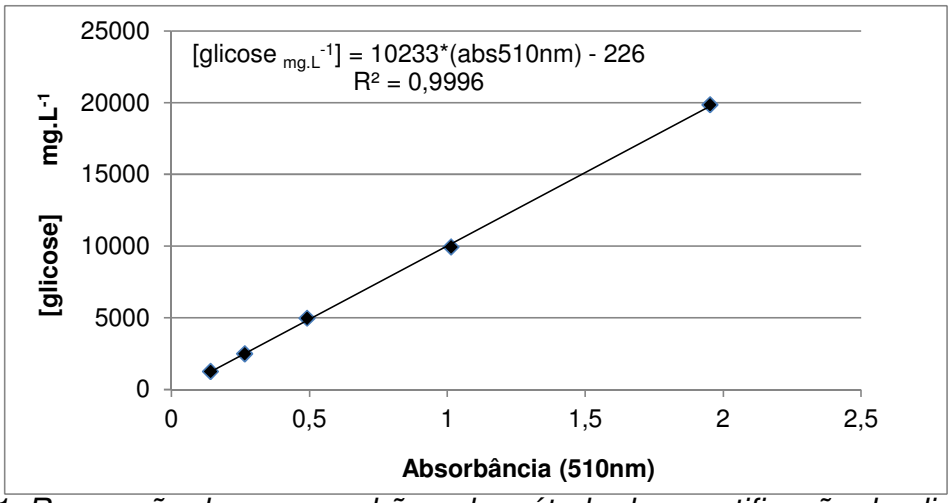

Figura 2.1. Regressão da curva padrão pelo método de quantificação de glicose com kit enzimático de glicose oxidase (parâmetros de reação utilizados para determinação da atividade de amiloglicosidase)

No decorrer dos experimentos posteriores, estas curvas foram refeitas sempre que preparados novos reagentes para realização dos ensaios, de forma a garantir a correta conversão dos valores de absorbância em concentração de açúcares. As curvas obtidas posteriormente para cada açúcar não são mostradas nesta tese, uma vez que o objetivo neste momento foi apenas ilustrar como as curvas padrão foram determinadas e como o pH afeta a inclinação das mesmas.

Após obter as curvas padrão para mensurar a atividade das enzimas, foram realizados os ensaios de atividade, utilizando-se diferentes concentrações de enzimas (α-amilase, glicose-oxidase, amiloglicosidase e protease neutra) e também variando-se a concentração de substrato e tempo de ação (β-galactosidase). O principal objetivo destes ensaios foi determinar concentrações de enzimas para obter leituras de absorbância adequadas, isto é, concentrações de enzimas que após a atividade resultassem em leituras de absorbância no meio da faixa das curvas padrão obtidas, de forma que fosse possível reproduzir as mesmas condições de ensaio após a HAP, mesmo que o processo promovesse aumento ou redução da atividade enzimática.

As metodologias de análises foram escolhidas em função de sua simplicidade, especificidade, rapidez de resposta e, principalmente, pela possiblidade de agrupamento de enzimas diferentes utilizando-se a mesma metodologia de análise,

de forma a reduzir o número de reagentes a serem adquiridos bem como facilitar a execução dos experimentos com enzimas. As *Tabelas 2.4, 2.5, 2.6, 2.7* e *2.8* mostram os resultados obtidos para a atividade de glicose oxidase, α-amilase, β-galactosidase, amiloglicosidase e protease neutra, respectivamente.

Tabela 2.4. Efeito da concentração inicial de glicose oxidase na atividade da enzima (atividade média ± desvio padrão)

[glicose-oxidase]	Absorbância 547nm	Atividade (U/g)
0,030 %	0,6058 0,6048 0,6086	$3,06 \cdot 10^6 \pm 0,4 \cdot 10^5$
0,015 %	0,6316 0,6346 0,6286	$2,56 \cdot 10^6 \pm 0,6 \cdot 10^5$
0,005 %	0,704 0,7398 0,7178	$6,86 \cdot 10^6 \pm 0,0 \cdot 10^5$
0,002 %	0,7498 0,7296 0,7399	$5,91 \cdot 10^6 \pm 0,0 \cdot 10^5$

A partir dos resultados obtidos estabeleceu-se a concentração de 0,03% como a ideal para determinação da atividade de glicose-oxidase. Isto porque, na determinação de glicose oxidase, o ideal é que se tenha a maior diferença possível entre a amostra contendo apenas glicose (cuja média de absorbância foi 0,7712) e a amostra cuja glicose foi consumida pela enzima (absorbância média de 0,6064). Assim, utilizando-se estes dois valores obtém-se uma diferença média de leitura de 0,1648, que é um valor razoável considerando-se a repetitividade e os desvios normalmente obtidos para o método enzimático e para leituras no espectrofotômetro.

Tabela 2.5. Efeito da concentração inicial de α-amilase na atividade da enzima (atividade média ± desvio padrão)

[α-amilase]	Absorbância 547nm	Atividade (U/g)
0,0250 %	> 1,0000 > 1,0000 > 1,0000	n.d.*
0,0100%	0,7025 0,7166 0,7091	$1,61 \cdot 10^5 \pm 0,2 \cdot 10^4$
0,0050%	0,6831 0,6708 0,6622	$2,97 \cdot 10^5 \pm 0,7 \cdot 10^4$
0,0010%	0,4697 0,4827 0,4744	$8,41 \cdot 10^5 \pm 2,2 \cdot 10^4$
0,0005%	0,4178 0,3928 0,4282	$1,27 \cdot 10^6 \pm 1,2 \cdot 10^5$

* não determinada pela leitura de absorbância ter ficado acima da faixa na qual foi estabelecida a curva padrão.

A partir dos resultados obtidos estabeleceu-se a concentração de 0,01% como a ideal para determinação da atividade de α-amilase considerando-se a faixa de leitura de absorbância intermediária e a curva padrão obtida para a maltose.

Tabela 2.6. Efeito da concentração de β-galactosidase e lactose e do tempo de reação na atividade da enzima (atividade média ± desvio padrão)

[β-galactosidase] - [lactose] - tempo (min)	Absorbância 510nm	Atividade enzimática (U/g)
0,5% - 2% - 15 min	0,3783 0,3658 0,361	$1,92 \cdot 10^3 \pm 0,7 \cdot 10^2$
0,5% - 3% - 15 min	0,6143 0,6331 0,6585	$3,85 \cdot 10^3 \pm 1,9 \cdot 10^2$
1% - 2% - 15 min	0,9779 0,9784 0,9762	n.d.*
1% - 3% - 15 min	1,5623 1,4955 1,5551	n.d.
0,5% - 2% - 30 min	0,3759 0,3867 0,4122	$1,00 \cdot 10^3 \pm 0,8 \cdot 10^2$
0,5% - 3% - 30 min	0,6322 0,6332 0,624	$1,83 \cdot 10^3 \pm 0,2 \cdot 10^2$
1% - 2% - 30min	0,9923 0,987 0,9697	n.d.
1% - 3% - 30 min	1,4408 1,3757 1,4449	n.d.

* não determinada pela leitura de absorbância ter ficado acima da faixa na qual foi estabelecida a curva padrão.

Diferentemente das demais enzimas avaliadas, a β-galactosidase teve o método de atividade determinado pela variação da concentração de enzima e substrato e também do tempo de contato para a reação. Isto porque a enzima estava mais diluída, requerendo maiores concentração para avaliação da atividade e porque métodos diferentes utilizaram variadas concentrações de lactose e tempo de contato. A partir dos resultados obtidos foram estabelecidas as concentrações de β-galactosidase de 0,5% e de lactose de 2%, e o tempo de ação de 15 minutos como condições ideais para determinação da atividade da enzima, considerando-se a faixa de leitura de absorbância que apresentou linearidade para a análise (entre 0,06 e 0,68).

Tabela 2.7. Efeito da concentração inicial de amiloglicosidase na atividade da enzima (atividade média ± desvio padrão)

[amiloglicosidase]	Absorbância 510nm	Atividade (U/g)
0,100 %	> 2,0000 > 2,0000 > 2,0000	n.d.*
0,050 %	1,6056 1,6172 1,6188	$5,65 \cdot 10^{7} \pm 2,51 \cdot 10^{6}$
0,010 %	1,1637 1,1154 1,1524	$1,48 \cdot 10^{8} \pm 3,06 \cdot 10^{6}$
0,005 %	0,7913 0,8560 0,7043	$2,02 \cdot 10^{8} \pm 1,99 \cdot 10^{7}$

* não determinada pela leitura de absorbância ter ficado acima da faixa na qual foi estabelecida a curva padrão.

A partir dos resultados obtidos estabeleceu-se a concentração de 0,01% como a ideal para determinação da atividade de amiloglicosidase considerando-se a faixa de leitura de absorbância e a atividade final obtida (maior atividade).

Tabela 2.8. Efeito concentração inicial de protease neutra na atividade da enzima (atividade média ± desvio padrão)

[protease neutra]	Δ Absorbância 275nm	Atividade (U/g)
0,250%	0,4697 0,4771 0,4736	$1,70 \cdot 10^4 \pm 1,33 \cdot 10^2$
0,125 %	0,2864 0,2938 0,2907	$2,09 \cdot 10^4 \pm 2,67 \cdot 10^2$
0,100 %	0,3411 0,3055 0,3274	$2,92 \cdot 10^4 \pm 1,62 \cdot 10^3$
0,050 %	0,2202 0,1567 0,1950	$3,43 \cdot 10^4 \pm 5,67 \cdot 10^3$

A partir dos resultados obtidos estabeleceu-se a concentração de 0,1% como a ideal para determinação da atividade da protease neutra, considerando-se a maior diferença entre a absorbância da caseína nativa e hidrolisada, o valor de absorbância absoluto da caseína hidrolisada (aproximadamente 0,5) e a atividade final obtida (maior atividade para amostras com um Δ_{abs} >0,3).

Assim, foram pré-estabelecidas as metodologias de avaliação das enzimas estudadas. Estas metodologias foram aplicadas antes e após a homogeneização à alta pressão para determinar como o processo afeta a atividade da enzima. A atividade máxima foi determinada nas condições ótimas (pH e temperatura) e a atividade residual foi determinada após as diferentes condições de processos avaliadas (pressão de homogeneização, temperatura e pH).

2.4. Conclusões

Concluiu-se que as metodologias escolhidas para avaliação da atividade enzimática foram simples e apresentaram boa repetibilidade. Os resultados obtidos demonstraram como o pH afeta a absorbância das amostras de açúcares, sendo muito importante o estabelecimento das curvas padrão nos valores de pH em que a atividade enzimática foi avaliada.

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Capítulo 3. High pressure homogenization of a fungi α-amylase

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Resumo

O efeito do processo de homogeneização à alta pressão (HAP) sobre a atividade e a estabilidade da α-amilase de *Aspergillus niger* foi estudado. Uma solução de enzima foi preparada em tampão acetato 0,1 M, pH 5,8 adicionado de 10 mM of $CaCl_2$ e posteriormente homogeneizada em pressões de até 1500 bar. A avaliação da atividade enzimática a 15, 45 e 75ºC após a homogeneização não mostrou nenhuma alteração. A avaliação do requerimento de cálcio na estabilidade da enzima durante a homogeneização também foi realizada, através do processamento da enzima em solução tampão com e sem adição de cloreto de cálcio. Os resultados demonstraram não haver diferença significativa ($p<0,05$) entre as amostras, indicando que a enzima é estável à homogeneização mesmo na ausência de cálcio. A estabilidade durante a estocagem refrigerada (8ºC) das amostras homogeneizadas em pH entre 4,0 e 6,7 foi medida pela avaliação da atividade da α-amilase a 15, 45 e 75ºC e os resultados mostraram que a atividade enzimática se manteve inalterada. A última tentativa de modificar a atividade da enzima foi a realização da HAP com elevada temperatura incial (65ºC), mas os resultados obtidos mais uma vez demonstraram que a enzima é resistente ao processo. Portanto, concluiu-se que a α-amilase é uma enzima altamente estável ao processamento por HAP.

Palavras-Chave: *α-amylase • ultra alta pressão de homogeneização • atividade enzimática*

Abstract

The activity and stability of α-amylase after high pressure homogenization were investigated. The enzyme buffer solution was processed at homogenization pressures up to 1500 bar. No changes in the enzymatic activity at 15, 45 and 75ºC were observed after the homogenization process. The evaluation of calcium requirement to preserve the α-amylase stability during homogenization was carried out and the results indicated that the enzyme was stable even with no calcium available. The stability during storage (4 days), at pH from 4.0 to 6.7 and at a temperature from 15 to 75ºC was also unaltered after homogenization. Additionally, the homogenization at elevated temperature (65ºC) was not able to change the α-amylase activity. Therefore, it was concluded that this enzyme is resistant to the high pressure homogenization process.

Key-words: *α-amylase • ultra-high pressure homogenization • enzymatic activity*

3.1. Introduction

High pressure homogenization (HPH) in an emerging technology applied to food preservation with a minimum sensory and nutritional damage (Tribst, Sant'ana, & de Massaguer, 2009a). This process was previously studied to inactivate vegetative bacterial (Campos, & Cristianini, 2007; Tribst, Franchi, & Cristianini, 2008) yeasts and molds (Tahiri, Makhlouf, Paquin, & Fliss, 2006, Tribst, Franchi, Cristianini, & de Massaguer, 2009b, Tribst, Franchi, de Massaguer, & Cristianini 2011). Moreover, some studies evaluated the HPH consequences in protein (Vannini, Lanciotti, Baldi, & Guerzoni, 2004, Vanini et al., 2008) and polysaccharides (Lacroix, Fliss, & Makhlouf, 2005). The effect of the HPH in enzymes was studied by few authors (Lacroix et al., 2005, Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009, Liu et al., 2009 a,b) and normally in specific substrates (Welti-Chanes at al., 2009, Liu et al., 2009 a,b). These studies showed that HPH was able to promote enzyme activation (Liu et al., 2009 a,b; Liu et al., 2010a) or inactivation (Lacroix et al., 2005, Welti-Chanes at al., 2009) and these effects were commonly associated to the type of enzyme and the applied pressure (Liu et al., 2009 a,b).

α-amylase (1,4-α-D-glucan glucohydrolase: EC 3.2.1.1) is a endoglucanase that catalyzes arbitrarily the cleavage of the internal α-(1,4) glycosidic linkage of starch and related polysaccharides into dextrins and oligosaccharides (Wong, & Robertson, 2003, Michelin et al., 2010). It is commercially applied in bakery, brewery, corn syrup and alcohol production, detergents and in the textile industry (Wong, & Robertson, 2003). This enzyme holds the maximum market share of enzyme sales with its major application in the starch industry as well as its oldest and well-known usage in bakery (Wong, & Robertson, 2003, Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003).

Additionally to these applications, the use of α-amylase is growing in juice industry, aiming to reduce the starch content of some beverages of banana (Cheirsilp, & Umsakul, 2008), apple (Ceci, & Lozano, 2002), passion fruit (Domingues, Junior, Silva, Madrona, Cardoso, & Reis, 2011) and ginkgo (Zhang, Wang, & Xu, 2007). Also, considering the large volumes of fruit processed and juice production, it is

common that unripe fruit (commonly having high content of starch) be mixed to ripe ones. Under these conditions fruit pulp contains starch in sufficient amounts to cause turbidity or even gelatinize during processing, requiring a pretreatment with amylases to guarantee juice stability (Ribeiro, Henrique, Oliveira, Macedo, & Fleuri 2010).

Chemically, the α-amylase is a metalloenzyme which contains at least one Ca^{2+} ion (Vallee, Stein, Summerwell, & Fischer, 1959, Gupta et al., 2003) in its structure. Some researches related that calcium plays an important role in the enzyme's thermal stability (Robyt, & French, 1963, Violet, & Meunier, 1989, Hmidet, Maalej, Haddar, & Nasri, 2010). On the other hand, other authors did not observe this calcium role in the enzyme stability (Laderman et al., 1993, Dong, Vieille, Savchenko, & Zeikus, 1997, Sajedi et al., 2005).

The aim of this research was to determine the effect of the HPH on the activity and the stability of a fungi α-amylase.

3.2. Material and Methods

3.2.1. Enzyme and enzymatic activity

A commercial α-amylase, produced through *Aspergillus niger* fermentation, was obtained from Prozyn Biosolutions ® (São Paulo, SP, www.prozyn.com). The enzyme was presented as a yellow powder, with optimum pH at 4.4-6.0, activity temperature ranging between 40 and 65°C, with optimum at 45°C. For assays, α-amylase solution was prepared at 0.01%.

Enzyme activity was measured through reducing sugar method of the 3,5-dinitrosalicyclic acid method (DNSA) described by Wong, & Robertson (2003) with few alterations: α-amylase was diluted in acetate buffer 0.1M (pH 5.8) added by 10 mM of $CaCl_2$. 750 µL of enzymatic solution was added to 750 µL of soluble starch solution (1%) in tubes. The reaction was carried out at 45ºC/ 10 minutes and after, 450 µL of DNSA solution was added into tubes followed by heating at 100ºC/ 5 minutes to stop the reaction. After, samples were added 4.05 mL of the buffer, totalizing 6 mL. The sample absorbance was measured at 547 nm in a

spectrophotometer DU 800 (Beckman Coulter ®, Brea, CA), and the activity was calculated through a maltose standard curve. The standard curve was obtained using 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mmol of maltose solution. The maltose solution reacted with DNSA and after samples absorbance was measured at 547 nm in triplicate. The standard curve was determined through linear regression of the maltose absorbance data ([maltose_concentration] = $0.923.abs_{547nm}+0.06$, with R^2=0.998). One enzyme unit (U) was defined as the amount of enzyme catalyzing the release of 1 µmol of reducing groups from soluble starch measured as maltose and per minute.

3.2.2. Optimum pH and temperature

The α-amylase activity was carried out at pH 4.0, 5.5, 5.8, and 6.7, using 0.1 M acetate buffer (pH 4.0 – 5.8) and 0.1M Tris-HCl buffer (pH 6.7). The effect of the temperature incubation was evaluated at 15, 45 and 75º C. The enzymatic activity was measured by the DNSA method, changing the pH and the temperature. The condition that presented higher activity (pH and temperature) was established as optimum with 100% of enzymatic activity. The residual activity was calculated using the *Equation 3.1*.

$$\text{Residual activity (\%)} = (\text{activity}_{non\_optimum\_conditions} / \text{activity}_{optimum\_conditions}) .100 \qquad (\textit{Equation 3.1})$$

3.2.3. High pressure homogenization

The assays were carried out on High-Pressure Homogenizer Panda Plus (GEA-Niro-Soavi, Parma, Italy). The equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 1500 bar.

A volume of 2 L of the α-amylase solution (0.1M acetate buffer, pH at 5.8 added 10 mM of $CaCl_2$) was introduced into the product inlet reservoir at room temperature (23º C). The solution was processed under 0, 400, 800, 1200 and 1500 bar (just one pass), then it was collected (50 mL) and immediately cooled in an ice

bath. The retention time at the homogenization temperature was ± 10 s. The sample temperature was measured using a type T thermocouple. Untreated α-amylase (native) solution was used as a control sample. The enzymatic activity was performed at 15, 45 and 75º C just after homogenization.

3.2.4. Calcium effect on α-amylase stability to homogenization

To evaluate the calcium effects on the α-amylase stability, a volume of 4 L of the α-amylase solution (0.1M acetate buffer, pH at 5.8) was prepared. Half of this solution was added by 10 mM of $CaCl_2$ and half was kept with no calcium. These α-amylase solutions were homogenized at 0 and 1500 bar (only one pass) and its activities were measured using the DNSA method just after the process. The DNSA method was carried out with and without addition of calcium (10 mM of $CaCl_2$). The activity of native enzyme was measured as a control sample.

3.2.5. α-amylase stability at different pH and during refrigerated storage

A sample of the α-amylase solution (0.1 M acetate buffer, pH at 5.8 added 10 mM of $CaCl_2$) was homogenized at 1500 bar (just one pass). After HPH, α-amylase activity was measured at pH 4.0, 5.5, 5.8, and 6.7. In addition, HPH α-amylase was stored refrigerated (8ºC) during 4 days. Then, α-amylase activity was measured at 15, 45 and 75º C. A native enzyme was used as a control sample for both pH and storage assays.

3.2.6. Inlet temperature homogenization effect on the α-amylase stability

A volume of 2 L of the α-amylase solution (0.1 M acetate buffer, pH at 5.8 added 10 mM of $CaCl_2$) was introduced into the product inlet reservoir at high temperature (65ºC) and homogenized under 1500 bar (just one pass). Just after HPH, the α-amylase activity was measured at 15, 45 and 75º C. A native enzyme was used as a control sample.

3.2.7. Statistical Analysis

The analysis of variance (ANOVA) was performed to compare the effects of the different treatments and the Tukey test was used to determine the difference of them at a 5% confidence level. Statistical analyses were carried out in STATISTICA 5.0 software (StatiSoft, Inc., Tulsa, Okla., U.S.A.). All of the tested conditions and determinations of the α-amylase activity were triplicated. The results were represented as the mean ± standard deviation.

3.3. Results and Discussion

3.3.1. Optimum pH and temperature

The α-amylase activity at different pH and temperatures is shown in *Figure 3.1*. The optimum α-amylase conditions, i.e., the condition at a higher activity, were determined as a pH 5.8 and a temperature of 45ºC. At this condition the activity was 37,293.5 U per gram of enzyme, which was considered as 100% of residual activity.

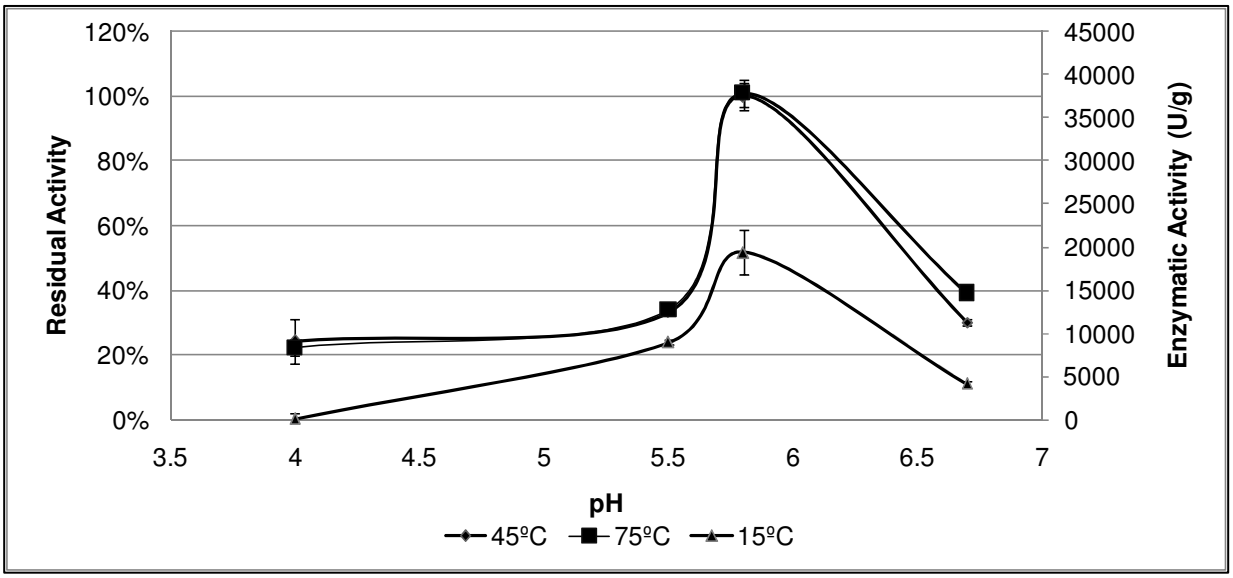


Figure 3.1. pH and temperature optima for the α-amylase activity

The pH variation resulted in significant differences in the enzymatic activity, reducing more than 50% when the enzymatic activity was evaluated at a pH of 5.5 or 6.7. On the contrary, the temperature variation promoted significant reduction of enzymatic activity only at 15º C. Therefore, this fungi α-amylase was relatively resistant to high temperature, being stable up to 75ºC. Higher temperatures were not evaluated since this enzyme is thermally inactivated at 80ºC/ 30 minutes, according to the enzyme supplier.

Considering the results showed in *Figure 3.1*, the measurement of the α-amylase enzymatic activity was evaluated at a pH of 5.8 after HPH. The temperatures of 15, 45 and 75º C were studied to evaluate if the high pressure homogenization affected the enzyme activity in non-ideal conditions.

3.3.2. High Pressure Homogenization of α-amylase

The HPH processes were carried out at pressures up to 1500 bar. The fast decompression during homogenization promotes intense shear and friction with consequent heating of the homogenized fluid. Considering that enzymes can be affected by heating, the sample temperature reached at each pressure was measured. The residence time at those temperatures was in the order of 10 s. *Table 3.1* shows the temperatures reached after homogenization.

Table 3.1. Temperature increase of the α-amylase solution during the HPH (inlet temperature = 23º C)

Pressure (bar)	Final Temperature (º C)	Temperature Increment (º C)
0	24.0	1.0
400	28.1	5.1
800	32.6	9.6
1200	35.3	12.3
1500	43.4	20.4

The pressure increase resulted in a linear temperature increment in the enzyme solution of around 1.2º C/100 bar; in addition it was observed that the maximum temperature was 43.4ºC at 1500 bar. This temperature was not enough to promote enzyme thermal denaturation and, consequently, all the effects observed after the HPH can be only attributed to the homogenization process. The effects of the HPH on α-amylase activity measured at different temperatures are shown in *Figure 3.2*.

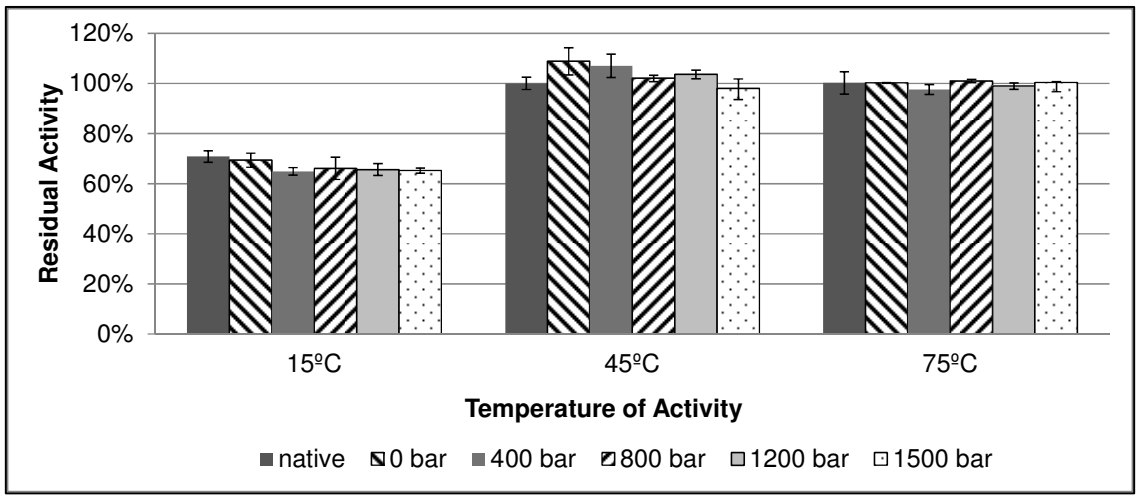


Figure 3.2. α-amylase activity at different temperatures after homogenization

The results showed no significant differences of enzymatic activity between the native and homogenized α-amylase up to 1500 bar ($p>0.05$), when the activity was measured at 15 and 75º C. At 45º C, a statistical difference ($p<0.05$) was observed between the α-amylase homogenized at 0 and 1500 bar; however, no difference was observed between these samples and the native α-amylase, indicating that the HPH promoted no significant activation or inactivation of the enzyme.

Previous results indicated that HPH promotes activation of poliphenoloxidase (Liu et al., 2009a,b) and inactivation of pectinmethylesterase (Lacroix et al, 2005, Welti-Chanes et al., 2009) at pressures in the same range studied in the present work. No previous work evaluated the effect of the HPH on α-amylase. The different effects of HPH on different enzymes may suggest that enzyme susceptibility to the process diverge according to their molecular structure and location of their active

sites. Moreover, it is important to highlight that the previous results were obtained for HPH of enzyme and substrate together (effects of HPH evaluated in fruit extracts), then, it is also possible that the homogenization process changed the substrate availability to the enzyme reaction.

Considering that no differences were observed between the evaluated pressures, the subsequent assays were carried out just with the native and the high pressure homogenized enzyme at 0 and/or 1500bar.

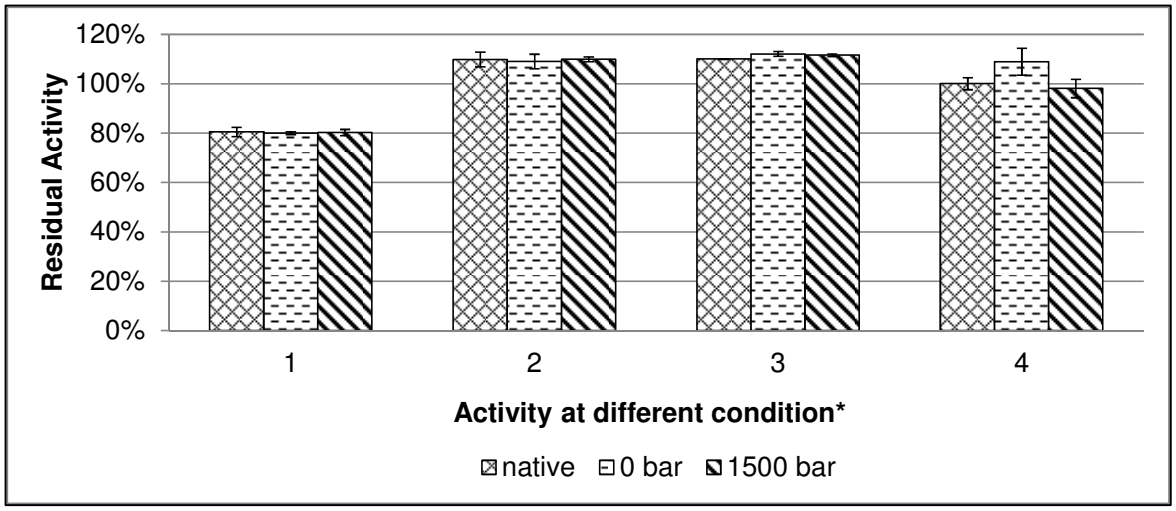
3.3.3. Calcium effect on α-amylase stability to homogenization and its requirements on measurement of enzyme activity

The α-amylase is a metalloenzyme and calcium was previously described as an important ion to guarantee the α-amylase stability (Robyt, & French, 1963, Violet, & Meunier, 1989; Hmidet et al., 2010). Thus, the calcium role in the α-amylase stability during and after HPH was evaluated. The results are shown in *Figure 3.3*.

The results showed no significant differences ($p > 0.05$) at the conditions # 1, 2 and 3. Just at the condition # 4 (With calcium in the enzyme solution and during the enzymatic activity measurement) a significant difference was observed between the α-amylase homogenized at 0 and 1500 bar. However, these samples were not different from the native α-amylase, indicating no enzymatic activity change after HPH. Additionally, only at the condition #1 (with no calcium in the enzyme solution and during enzymatic activity measurement), the enzyme activity was significantly different from the other evaluated conditions, even for the native enzyme, with ± 20% reduction in the enzymatic activity.

These results indicate that the α-amylase high stability to the HPH cannot be attributed to calcium addition in the enzyme solution. On the other hand, the results corroborated the Ca^{2+} requirement to reach the maximum activity of the enzyme during activity measurement (Violet, & Meunier, 1989). The calcium effect on α-amylase activity/stability was linked to the enzyme source, being essential to improve thermal stability (Robyt, & French, 1963, Violet, & Meunier, 1989; Hmidet et al., 2010)

or activity in some cases (Violet, & Meunier, 1989) and dispensable in others (Laderman et al., 1993; Dong et al., 1997; Sajedi et al., 2005).

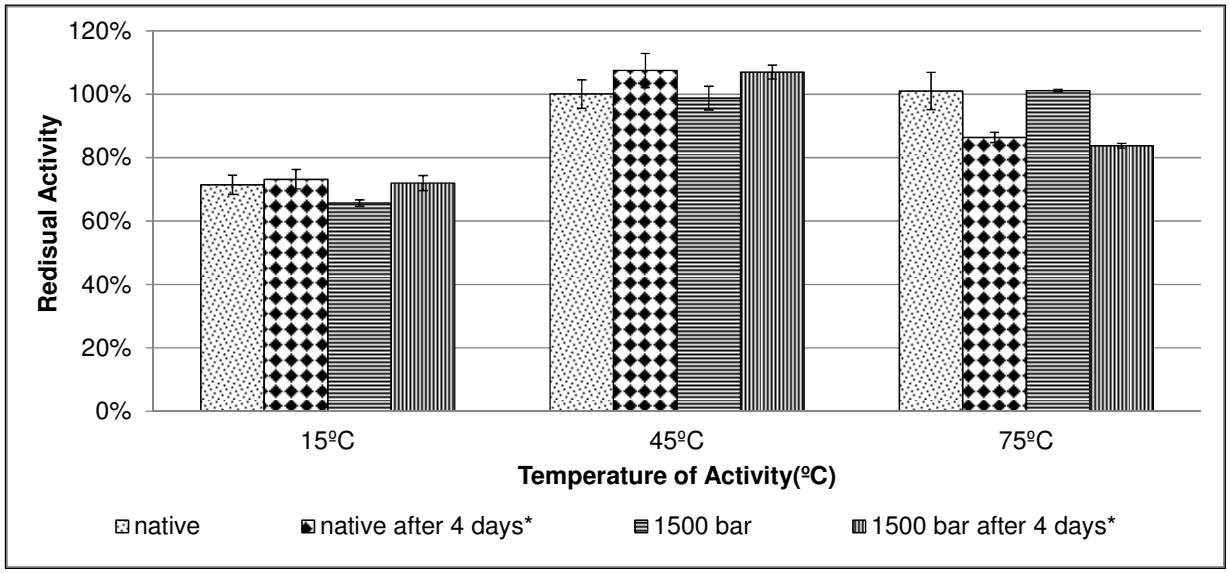


**Condition#1: with no calcium in the enzyme solution and during enzymatic activity measurement. Condition #2: with no calcium in the enzyme solution but with calcium during the enzymatic activity measurement. Condition #3: With calcium in the enzyme solution but with 50% of calcium during the enzymatic activity measurement. Condition #4: With calcium in the enzyme solution and during the enzymatic activity measurement.*

Figure 3.3. Calcium effect of the α-amylase stability on homogenization and its requirements for enzyme activity measurement

3.3.4. α-amylase stability at different pH and during refrigerated storage

The stability of high pressure homogenized α-amylase during storage and at different pH was evaluated. Previous results showed that although HPH was not able to change the enzymatic activity, it was able to stabilize trypsin to thermal denaturation (Liu et al., 2010a). No changes in the α-amylase thermal stability were expected since no differences were found between the native and the homogenized enzyme activity at 75º C (*Figure 3.1*). The effect of the HPH in the enzyme stability after storage at 8º C (4 days) is shown in *Figure 3.4* and the HPH effect in the α-amylase pH stability is shown in *Figure 3.5*.



** Enzyme diluted in an acetate buffer 0.1M (pH 5.8) 10 mM of $CaCl_2$ and storage for 4 days (8 °C).*

Figure 3.4. Effect of refrigerated storage on the stability of the HPH α-amylase

The results indicated that the enzyme solution refrigerated during 4 days preserved its initial activity at 15 and 45º C, with no significant differences from the freshly prepared enzyme ($p>0.05$). Additionally, no significant differences were found between the native and the HPH enzyme, indicating that homogenization has not reduced nor increased the enzyme stability during storage in the buffer at 8° C.

On the contrary, at 75º C a significant activity reduction (±15%) was observed after storage of native and homogenized samples (equal activity reduction was observed for both samples). This indicates that enzyme storage in buffer at low temperature reduces the α-amylase activity at high temperature and that HPH did not change this effect of storage. Previous results obtained by Liu et al. (2010b) indicated an intense papain activity loss of high pressure homogenized sample after 24h of storage, indicating that papain is less stable than the α-amylase after the HPH process.

The activity of high pressure homogenized α-amylase at different pH presented similar results to the previously obtained for the native enzyme (*Figure 3.1*), with a maximum activity at a pH of 5.8 and with same activity at the pH of 4.0, 5.5 and 6.7.

Therefore, no differences were observed between the native and the homogenized α-amylase at each evaluated pH. Consequently, the HPH did not change the activity and/or the stability of the studied fungi α-amylase.

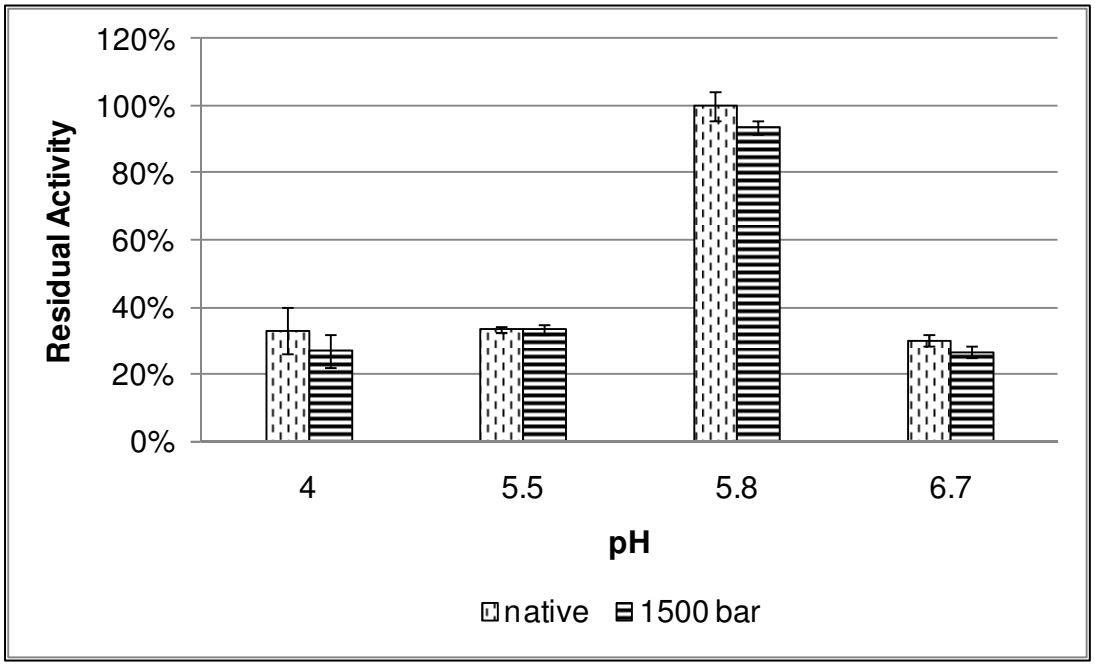


Figure 3.5. Effect of the homogenization on the α-amylase pH stability

3.3.5. Inlet temperature homogenization effect on the α-amylase stability

The association of homogenization and heat was also tested, carrying out the homogenization process at high temperature (65º C). The results of this assay are shown in *Figure 3.6*.

The combination of the HPH and a high inlet temperature again promoted no changes in the α-amylase activity. Thus, it can be determined that the HPH at the evaluated conditions was not able to promote significant changes in the α-amylase. This result can be useful for the industry that intent to use HPH with products containing α-amylase, since the results indicate, with no doubts, that the homogenization did not affect the activity and stability of the enzyme. This is mainly interesting to some juice industries that apply α-amylase for juices clarification and viscosity reduction (Ceci, & Lozano, 2002; Zhang, Wang, & Xu, 2007; Cheirsilp, & Umsakul, 2008, Ribeiro, et al., 2010; Domingues et al., 2011) and can use HPH as a

non thermal process to stabilize juices microbiologically and physically, through particle size reduction.

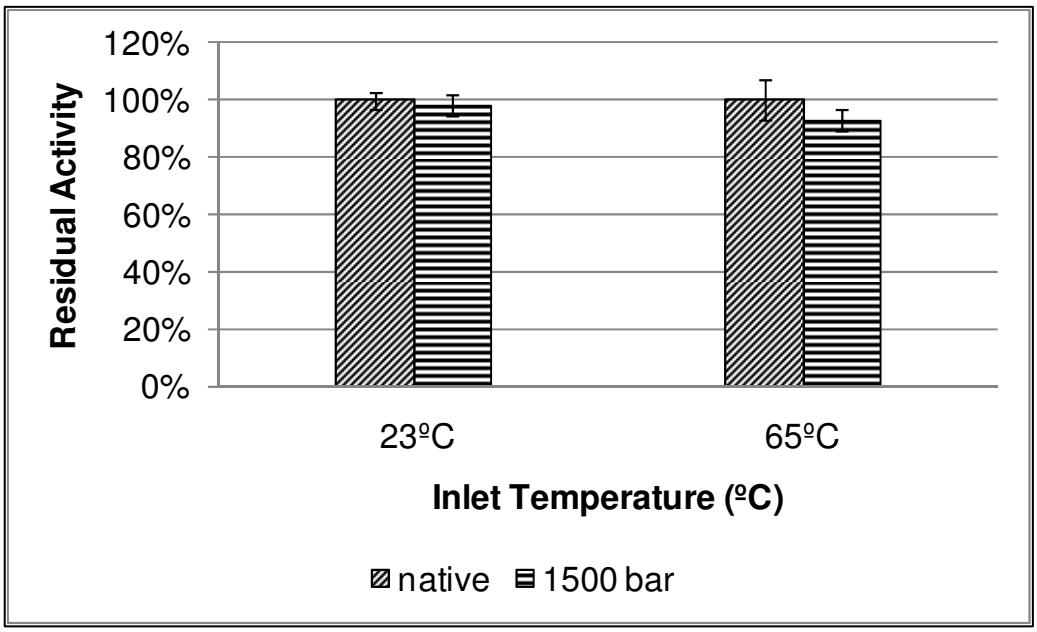


Figure 3.6. Effect of homogenization at a high inlet temperature on the α-amylase

3.4. Conclusion

The α-amylase activity and stability was not affected by the high pressure homogenization up to 1500 bar and the homogenization at a high temperature also promote no changes in the enzyme activity. Therefore, it can be concluded that the fungi α-amylase is stable to high pressure homogenization up to 1500 bar.

Acknowledgements

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Capítulo 4. The effect of the high pressure homogenization on the activity and stability of a commercial neutral protease from *Bacillus subtilis*

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Resumo

Este estudo teve como objetivo avaliar o efeito da homogeneização à alta pressão (HAP) sobre a atividade de uma protease neutra comercial obtida por fermentação de *Bacillus subtilis.* A enzima foi processada a pressões até 2000 bar e a atividade residual medida entre 20 e 70ºC imediatamente após a homogeneização e após a estocagem refrigerada das amostras por 7 dias. Adicionalmente, o efeito da homogeneização à altas temperaturas foi avaliado. Quando a atividade foi medida logo após a HAP, os resultados não demonstraram aumento de atividade da protease a 55 ou 70ºC. Por outro lado, quando a atividade foi medida a 20ºC, um aumento considerável de atividade (~30%) foi observado após homogeneização a 2000 bar. Assim, é possível concluir que a homogeneização modificou a temperatura ótima de atividade da protease de 55 para 20ºC. Por outro lado, a homogeneização à alta temperatura reduziu três vezes a atividade a 20ºC, apesar de não ter causado alterações significativas a 55ºC. Estes dados sugerem que a HAP modifica a configuração da protease, uma vez que o mecanismo chave-fechadura é estritamente dependente da estrutura espacial da enzima. Além disso, as alterações ocorridas podem ou não ser permanentes, dependendo da pressão de homogeneização, temperatura da amostra na entrada do equipamento e das condições de estocagem da enzima, portanto, a HAP é um método promissor para modificação das características de proteases.

Palavras-chave: protease; homogeneização à alta pressão; atividade enzimática

Abstract

The activity of a commercial neutral protease from *Bacillus subtilis* after high pressure homogenization (HPH) was investigated. The enzyme was processed up to 2000 bar and the residual activity was measured from 20-70ºC during refrigerated storage. Moreover, the effect of HPH at high temperatures was evaluated. No improvement in the activities at 55-70ºC were observed after HPH, while an increase of ~30% in the 20ºC-activity was reached after 2000 bar processing. Thus, HPH shifted the optimum temperature from 55ºC to 20ºC. The high temperature homogenization caused no changes in 55ºC-activity, but reduced 20ºC-activity three times. It suggests that HPH modifies the protease configuration, changing enzyme performance (maximum activity condition), as the efficacy of lock-and-key mechanism is strictly dependent on enzyme spatial structure. The changes can be permanent or not, depending on homogenization pressure, inlet temperature and enzyme storage conditions. Therefore, the HPH is a promising method to change protease characteristics.

Keywords: protease; high pressure homogenization; enzymatic activity

4.1. Introduction

The high pressure homogenization (HPH) emerged as a non-thermal technology in order to guarantee food safety, stability with a reduced sensory and nutritional damage (Tribst et al 2009a). This process was extensively studied to inactivate vegetative bacterial (Campos and Cristianini 2007, Tribst *et al.* 2008), yeasts and molds (Tahiri *et al.* 2006, Tribst *et al.* 2009b, Bevilacqua *et al.* 2011, Tribst *et al.* 2011), as well as to evaluated the effect of the homogenization in macromolecules, such as proteins (Vannini *et al.* 2004, Vanini *et al.* 2008) and polysaccharides (Lacroix *et al.* 2005). The effect of the HPH in enzymes was studied by some authors (Lacroix *et al.* 2005, Welti-Chanes *et al.* 2009, Liu *et al.* 2009 a,b, Liu *et al.* 2010a,b) and normally in specifics substrates (Welti-Chanes *et al.* 2009, Liu *et al.* 2009 a,b). The HPH can promote activation (Liu *et al.* 2009 a,b, Liu *et al.* 2010a) or inactivation (Lacroix *et al.* 2005, Welti-Chanes *et al.* 2009) of enzymes and the effect is normally associated to the enzyme and the applied pressure (Liu *et al.* 2009 a,b).

Proteases are enzymes that catalyze hydrolytic reactions in which protein molecules are degraded to peptides and amino acids (Sumantha *et al.* 2006). They are constituted of a very large and complex group of enzymes, which differ in properties such as the substrate specificity, the active site, and the catalytic mechanism, the optimum pH, the temperature and stability profile (Sumantha *et al.* 2006). Proteases can be sub-classified as carboxyl protease, cysteine protease, metallo protease, and serine protease, according to their activity sites and sensitivity to several inhibitors (Hartley, 1960). The proteases are one of the most important enzyme groups and represent up to 60% of the total enzyme sales, mainly used in detergents, meat, beer, animal feed, leather, and dairy industries (Esakkiraj *et al.* 2009). Specific applications of neutral proteases include milk protein modification, nitrogen control, mash extraction, and chill-haze removal in brewing, soy modification for the use as flavors, and in animal feeds (Schallmey *et al.* 2004), as well as silvering the recovery from photographic films with gelatin hydrolysis, cleaning processes, biopolishing of wool fabrics, and protein synthesis (Sumantha *et al.* 2006).

The effect of the HPH was previously studied in some proteases, such as trypsin (Liu *et al.* 2010a) and papain (Liu *et al.* 2010b). The effects on trypsin, which is a serine protease, showed that the homogenization at 80 MPa enhances the thermal stability of the enzyme, although not showing any effect in its activity (Liu *et al.* 2010a). The effects on papain, which is a neutral protease, indicated a slow and continuous activity reduction when homogenized between 120 and 180 MPa, and a reduction in its stability during 24 hours of storage (Liu *et al.* 2010b). There is any study in the literature regarding the effect of HPH on a *Bacillus subtilis* neutral protease. As the processing effect can be different for each enzyme, it is important to evaluate the *B. subtilis* neutral protease. Therefore, the aim of this study was to determine the effect of the HPH on the activity and stability of a neutral protease from *Bacillus subtilis*.

4.2. Material and Methods

4.2.1. Protease and enzymatic activity

The commercial neutral protease (Prozyn Biosolutions®, São Paulo, SP, www.prozyn.com, activity 5017 NU/g) used in this study was obtained as a product of the *Bacillus subtilis* fermentation. It is a metalloprotease with molar mass between 19-37 kDa, optimum pH close to 7.0 and optimum temperature above 50ºC.

The enzyme was evaluated at a concentration of 0.1% (w/v) in buffers, and the enzymatic activity was determined following the method described by Merheb *et al.* (2007) with few changes: the enzymatic solution was prepared in a 0.1 M phosphate buffer at a pH of 7.5. 200 µL of enzymatic solution was added to 400 µL of the same buffer mixed with 400 µL of casein (97.5% of purity, Synth, Brazil) solution at 0.5% (w/v) prepared in the same buffer. The reaction was conducted at 55ºC/30 minutes and then added 1 mL of 10% (w/v) trichloroacetic acid (TCA) to stop the reaction. The samples were centrifuged at 10,000 rpm/ 5 min/ 10ºC and its absorbance was measured at 275 nm in an UV-VIS spectrophotometer DU 800 (Beckman Coulter ®,

Brea, CA, USA). One unit of enzymes was defined as the amount of enzymes required to increase 0.1 in absorbance at 275 nm, under the assay conditions. The control samples were performed adding the TCA in the test tubes before the enzymatic solution, and the ΔAbs_{275nm} was determined through the absorbance differences of the sample and the control. The enzymatic activity was calculated according to *Equation 4.1*.

$$U/g = \Delta Abs_{275nm} \cdot 10 \cdot \text{dilution factor} / (0.2) \qquad (\textit{Equation 4.1})$$

4.2.2. Protease activity at different pH, temperatures and after 48h of storage

The protease activity was evaluated at a pH of 4.0 (below the enzyme stability), at 5.5 (expected as higher stability), and at 7.5 (expected as higher activity). The buffers used were 0.1 M of acetate (pH 4.0 – 5.5) and 0.1 M of phosphate (pH 7.5). The same molar buffer concentration was applied to avoid different changes caused by enzyme and buffer interaction.

The effect of the temperature was evaluated at 20°C (below the optimum), at 55°C (expected as optimum), and at 70ºC (expected as inactivation temperature). The condition at a higher activity (pH and temperature) was determined as the optimum, being 100% of the enzymatic activity. The residual activity was calculated according to *Equation 4.2*.

$$\text{Residual activity (\%)} = (\text{Activity at non ideal conditions} / \text{optimum activity}) \cdot 100 \qquad (\textit{Equation 4.2})$$

To evaluate the enzyme stability in solution during storage at 8ºC for 48 h, the protease was diluted at pH 4.0, 5.5 and 7.5 and then storage. After 48 h, the protease activity was measured at 20, 50 and 70ºC using the same pH of enzyme during storage. The enzymatic activity was determined by the method described in section 4.2.1, changing the pH and the temperature of activity measurement

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4.2.3. High pressure homogenization

A High-Pressure Homogenizator Panda Plus (GEA-Niro-Soavi, Parma, Italy) was used. The equipment contains a single intensifier pump that amplifies the hydraulic pressure up to 2000 bar. The flow rate is fixed at 9 L/h.

A total of 2 L of protease solution 0.1% (w/v) (90.96 U/mL) were prepared using phosphate buffer 0.1 M (pH 7.5). Then, protease solution was introduced into the product inlet reservoir at room temperature (23ºC) and processed under pressures of 0, 500, 1000, 1500, and 2000 bar. Solution was collected (50 mL) and immediately cooled in an ice bath. The sample temperature was measured after processing by a type T thermocouple. As a control sample, there was used unprocessed (native) protease solution. The enzymatic activity was performed at 20, 55, and 70ºC just after homogenization.

4.2.4. UV-Absorption spectra analysis of native and homogenized protease

The UV-absorption spectra of 0.1% of protease solution at pH 4.0, 5.5 and 7.5 before and after homogenization at 0, 500, 1000, 1500, and 2000 bar was measured in a UV–VIS spectrophotometer DU 800 (Beckman Coulter ®, Brea, CA). The UV absorption spectra were scanned from 200 to 400 nm (Liu *et al.* 2010a) to determine the absorption peak value and its wavelength.

4.2.5. Enzymatic stability during refrigerated storage

The stability of protease homogenized at 2000 bar during storage at 8ºC was evaluated by determining its activity at 20ºC and 55ºC. The assays were carried out after 0, 1, 4 and 7 days of storage. A sample of native enzyme was used as controls.

4.2.6. High inlet temperature homogenization effect on the protease activity and stability

The protease solution was homogenized at 0 and 2000 bar, as described in the item 4.2.3, but using inlet temperature of 60ºC. When sample is processed by HPH, the intense shear and friction involved dissipate mechanical energy in thermal energy, increasing product temperature (Pinho et al., 2011). Therefore, the high inlet temperature was set to 60ºC as larger temperatures would thermal denaturate the enzyme during processing.

The enzymatic activity was performed at 20 and 50ºC, just after homogenization and after one day of storage. The native sample was used as the control.

4.2.7. Statistical Analysis

The analysis of variance (ANOVA) was performed to compare the effects of the different treatments and the Tukey test was used to determine the difference of them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 5.0 software–(StatiSoft, Inc., Tulsa, Okla., U.S.A.). All process and protease activity determination were triplicated. The results were represented as mean ± standard deviation.

4.3. Results and Discussion

4.3.1. Enzyme characterization

The protease stability at different pH, temperatures and storage is shown in *Figure 4.1.*The optimum protease conditions, i.e., the condition at a higher activity, were determined as a pH 7.5 and a temperature of 55ºC. At this condition the activity was 90,963.3 U/g which was considered as 100% of residual activity. The maximum

activity was determined at the conditions described by the enzyme supplier (Prozyn Biosolutions®) as being ideal.

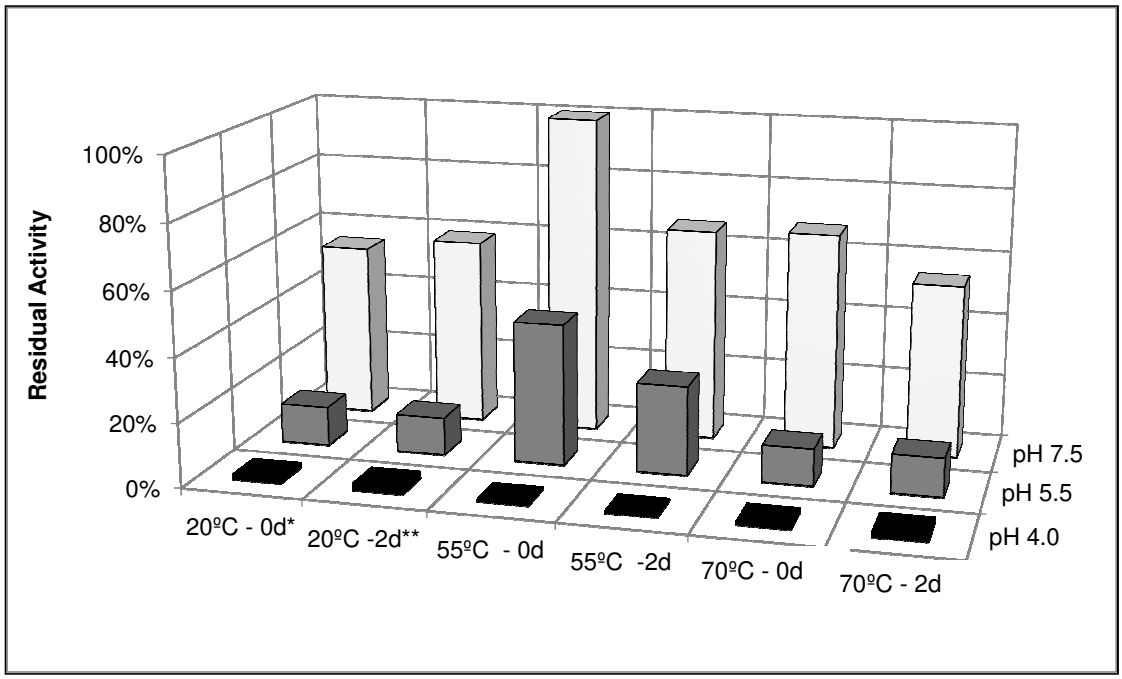

Figure 4.1. Protease activity at pH 4.0, 5.5 and 7.5 measured at 20, 55 and 70ºC just after enzyme solution preparation (*) in buffer at 0.1M and after 2 days of enzyme solution storage at 8ºC (**)

The change of the pH resulted in significant differences in the enzymatic activity, reducing more than 50% when the activity was measured at pH 5.5. At pH 4.0, there was no activity, which was expected since this pH is out of the pH stability range described by the enzyme supplier (Prozyn Biosolutions®). The not-optimum temperature also promoted a reduction in the enzymatic activity close to 40% at pH 7.5 and 50% at pH 5.5. The statistical evaluation of the activity loss at non-ideal temperatures showed significant differences between the results obtained for enzymes at pH 7.5 and 5.5. This demonstrated that at pH 7.5 the enzyme has a higher activity, even at non optimum conditions.

The statistical analysis indicated that the protease stability during storage was dependent on the pH and the temperature. At 20ºC, no loss of activity was observed

for all pH, which can indicate that the storage at 8ºC favored the enzymatic activity maintenance at low temperature. The maximum residual activity was obtained at 55ºC and pH 7.5 (same conditions observed for fresh enzymes). However, comparing the activities of fresh and stored enzyme at same condition, the highest activity loss occurred at 55ºC, indicating that the enzyme fraction that has higher activity also presented high instability during storage. At 70ºC, the activity loss was significant only at pH 7.5, around 20% of reduction. This suggested that, if the use of the enzyme after storage is carried out at a high temperature, the protease is more unstable when stored refrigerated at pH 7.5 than at pH 5.5. Therefore, this is the only condition where the protease at pH 7.5 showed higher instability than at pH 5.5.

Considering that the enzyme higher activities were at pH 7.5, the HPH process was carried out at this pH, aiming to maximize the differences of the protease processed at different conditions.

4.3.2. High pressure homogenization of protease

The HPH processes were carried out at pressures up to 2000 bar. The fast decompression during homogenization promotes intense shear and friction with consequent heating of the homogenized fluid. Considering that enzymes can be affected by heating, the sample temperature reached at each pressure was measured. The residence time at those temperatures was in the order of 10 s. *Table 4.1* shows the temperatures reached after homogenization.

Table 4.1.Sample temperature increasing during HPH (inlet temp. = 23º C)

Pressure (bar)	Final Temperature (º C)	Temperature Increment (º C)
0	26.0	3.0
500	32.4	9.4
1000	38.1	15.1
1500	44.5	21.5
2000	47.2	24.2

The pressure increment resulted in a linear temperature increment in the enzyme solution of around 1.1º C/100 bar; in addition it was observed that the maximum temperature was 47.2ºC at 2000 bar. This temperature is too low to promote enzyme thermal denaturation and, consequently, all the effects observed after the HPH can be only attributed to the homogenization process. The effects of the HPH on protease activity measured at different temperatures are shown in *Figure 4.2*.

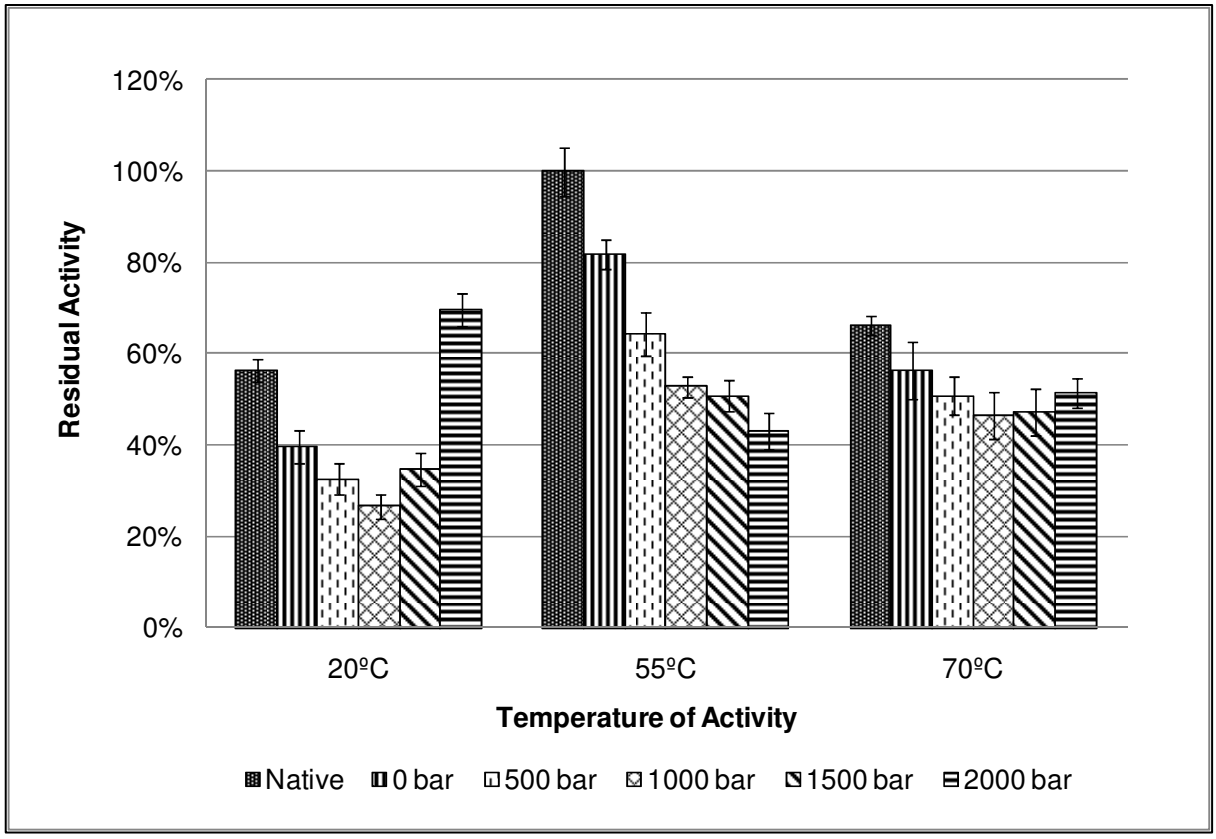

Figure 4.2. Effects of the HPH (between 0 and 2000 bar) on the protease activity measured at 20, 55 and 70ºC

The assays were triplicated and no statistical difference ($p>0.05$) was observed in the processed samples at the same conditions, indicating a good repeatability of the process. No statistical differences were determined in the activity of native protease and homogenized at 0 bar when activity was measured at 20°C and at 70ºC, and higher activities were obtained at 55ºC. It demonstrates that there are

small changes in enzyme configuration only due to pumping it through the equipment. Moreover, although those changes are not significant to the activity at 20°C and 70ºC, even those small changes can slight affect the enzyme activity at 55°C.

After the HPH at 500 bar, the protease presented distinct activity at each evaluated incubation temperature ($p<0.05$) and, after 1000 bar, the intense reduction of protease activity at the optimum temperature equaled the enzymatic activity measured at 55 and 70ºC. At 2000 bar, an increase was observed in the activity measured at 20°C, becoming the optimum temperature after homogenization at 2000 bar.

The evaluation of the results indicated that HPH effects on protease were dependent on the temperature of enzymatic activity. At the optimum temperature (55ºC), a rapid reduction on protease activity was observed between 0 and 1000 bar, followed by a slight reduction between 1000 and 2000 bar. In addition, a significant reduction was observed after homogenization at 0 bar, showing that even the protease solution pumping into the equipment is enough to promote small changes in enzyme configuration due to shear stress. The difference between the inactivation level reached at the earlier 1000 bar and between 1000 and 2000 bar suggested that the protease is unstable and low pressures probably promoted changes in its active sites configuration. After this first modification, the enzyme became more stable to the effects of homogenization.

The protease homogenization between 500 and 2000 bar resulted in a slight but significant reduction of enzymatic activity at 70ºC, when compared to native enzymes. However, no statistical differences were observed between the reductions obtained at different homogenization pressures. The activity loss after homogenization was lower when the enzyme activity was measured at 70ºC than at 55ºC, indicating that the enzyme stability was better in non-optimum conditions. Previous results obtained for homogenized trypsin showed an increase of its thermal resistance after homogenization (Liu *et al.* 2010a). However, in the present study, the results obtained at 70ºC showed no increment in the thermal resistance after processing, indicating that the process effects are different for each evaluated enzyme.

The enzyme activity reduction at 20^0C was observed in homogenization pressures up to 1000 bar, being similar to the results obtained at 55ºC. However, at higher pressures, an increase in the enzymatic activity occurred. The activity after enzyme homogenization at 2000 bar was around 30% higher than the native enzymes at this temperature, showing that the process promoted changes in the enzyme molecule that enhanced its activity at 20ºC.

The results suggest that, although the maximum activity is reached at 55ºC for native enzyme, this is not a stable condition when compared to the protease activity measured at 20ºC or 70ºC, since at 55ºC the highest activity loss happened when the protease was stored in a buffer system at 8ºC or when it was homogenized.

Considering the results obtained by other authors, the HPH was described as a process able to activate (Liu *et al.* 2009a,b), inactivate (Welti-Chanes *et al.* 2009) or promote no changes in enzymatic activity (Liu *et al.* 2010a), being this results obtained for different enzymes processed at similar conditions to those described in this paper. This demonstrates that the effect of homogenization varies for each enzyme and may be affected by the homogenization conditions.

The HPH is known as a process able to promote enzyme denaturation, being able to change the enzyme structure, including the secondary one (α-helix, β-sheet and β-turn) (Liu et al., 2009b, 2010). These conformational changes modify the protease molecular structure and could alter the optimum temperature to enzyme activity (which can be explained as the ideal temperature to improve the lock and key mechanism between casein and protease, considering the new molecular conformation due to spatial enzyme alteration caused by homogenization). Therefore, the results obtained for the homogenized enzyme in this paper highlights that this process can change the optimum temperature for enzyme activity, as observed for the protease homogenized at 2000 bar.

4.3.3. UV-Absorption spectra analysis of native and homogenized protease

The enzyme changes due to the HPH were evaluated through UV-absorption spectra analysis of the homogenized protease at pH 4.0, 5.5 and 7.5 at room

temperature. Changes in enzyme's UV absorption after homogenization at different pH can indicate that the process was able to promote changes in the protein molecule structure (Liu *et al.* 2010a) as exposure of hydrophobic residues of tyrosine and tryptophan (Liu *et al.* 2009). The results of UV-absorption between 250 and 285 nm (peak region) are shown in *Figure 4.3*.

The obtained results showed no differences in the wavelength peak absorption (275 nm) and in the maximum absorption between the native and the homogenized (0-2000 bar) enzyme. Further, no significant differences were observed between the samples homogenized at different pH.

Considering the results obtained for protease activity after HPH (*Figure 4.2*), no correlation was observed with the UV-absorption spectra, indicating that UV-absorption cannot be used as an indirect way to evaluate if HPH was able to promote changes in the evaluated protease. Also, it is possible that the other changes caused by HPH on enzyme structures (i.e. partial denaturation of tertiary and secondary structure) are enough to change the protease activity.

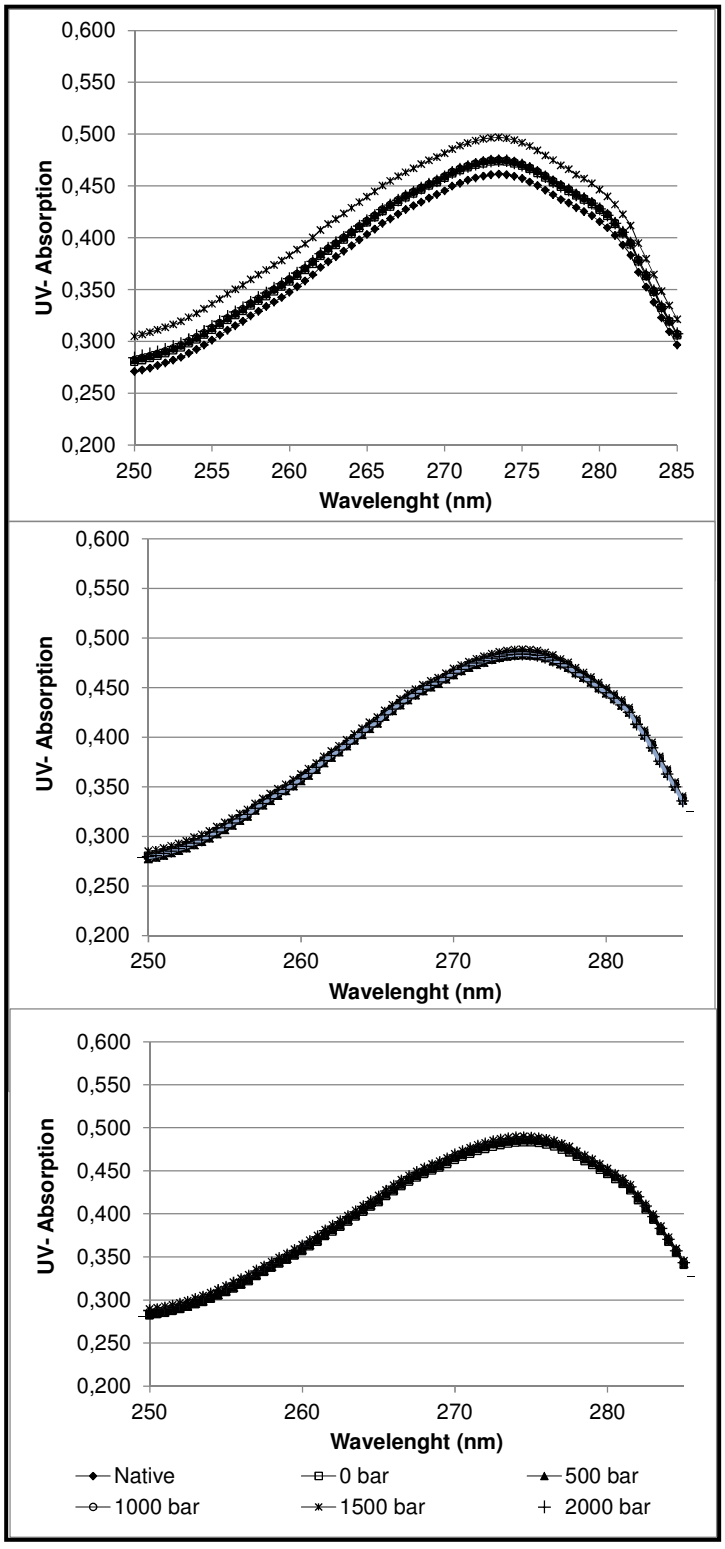

Figure 4.3. Effects of the HPH at pH 4.5 (A), 5.5 (B) and 7.5 (C) on the protease UV-absorption spectra between 200 and 400 nm

4.3.4. Stability during refrigerated storage of homogenized protease

The enzyme stability during refrigerated storage was carried out in order to evaluate if changes induced by the HPH processing were permanent or reversible. Results are shown in *Figure 4.4.*

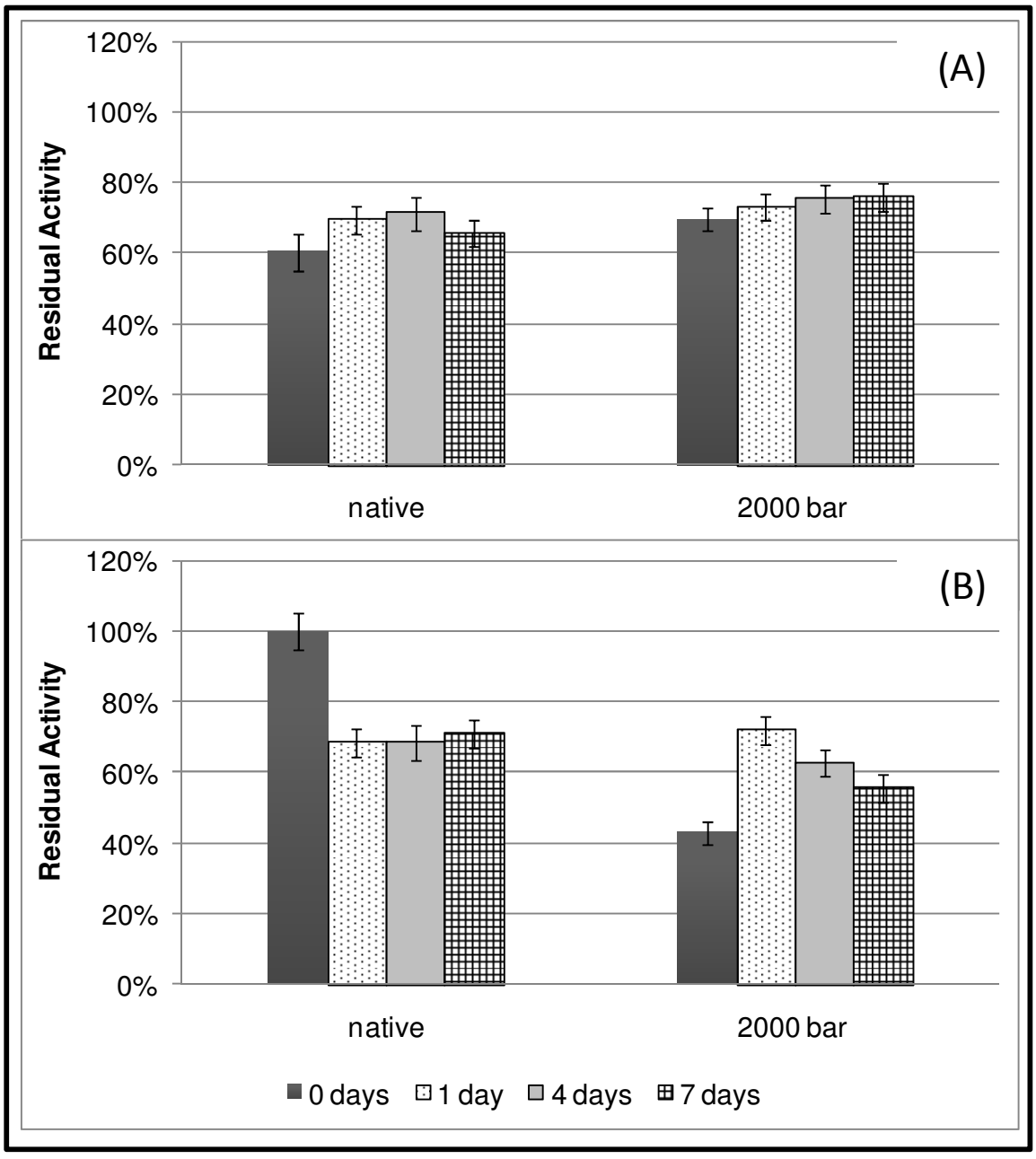

Figure 4.4. Stability of native and high pressure homogenized (2000 bar) protease stored at pH 7.5 and 8ºC for one week. Activity measured at 20ºC (A) and 55ºC (B)

The protease activities were evaluated at 55ºC and 20ºC due to their higher activity changes just after homogenization. The effect of storage was different for enzyme activity measured at 55ºC and 20ºC. For the native enzyme activity measured at 55ºC, the reduction was statistically significant in the first 4 days, being more intense on the first day.

The homogenized enzyme, in contrast, showed an activity increment (up to 30%), being statistically equal to the native enzyme after one day of storage. This clearly demonstrates that protease inactivation by the HPH was reversible.

The native enzyme activity at 20ºC increased around 10% on the first day; after, no significant differences were observed until the end of the storage period. The homogenized enzyme, in contrast, showed a slight activity increase during storage, but it was not statistically significant. The increase of protease activity at these conditions could be attributed due to isoenzymes arising throughout the storage (Richardson and Hyslop, 1985). Welti-Chanes *et al.* (2009) observed high increase in the activity of homogenized pectinmethylesterase during storage and attributed this phenomenon to the homogenization ability to split isoenzymes that could react later on in the food system. This effect, however, was not observed for protease, since the activity of native and homogenized protease during storage was the same ($p>0.05$).

Also, is interesting to evaluate that the native protease activity at 20 and 55^{o}C after storage at 8^{o}C showed a significant activity reduction at 55^{o}C, and a slight activity improvement at 20^{o}C. This phenomenon can be attributed to the low temperature (8°C) of storage, which may improve the activity of native protease at relative low temperatures (20ºC) in detriment of high temperatures (55ºC).

4.3.5. Inlet temperature homogenization effect in the protease activity and stability

To evaluate if the homogenizer inlet temperature was able to change the effects of HPH on protease, this assay was performed at pH 7.5 and it was evaluated at pressures of 0 bar (atmospheric temperature) and 2000 bar. The 0 bar was chosen to evaluate if the increment in the inlet temperature was enough to improve the

enzyme inactivation and 2000 bar was chosen to evaluate the effects of maximum pressure of the homogenizer. Also, considering that the main changes in protease during storage occurred at the first day, protease homogenized at 60 ºC inlet temperature was evaluated just after homogenization and after one day of storage. These results are shown in *Figure 4.5*.

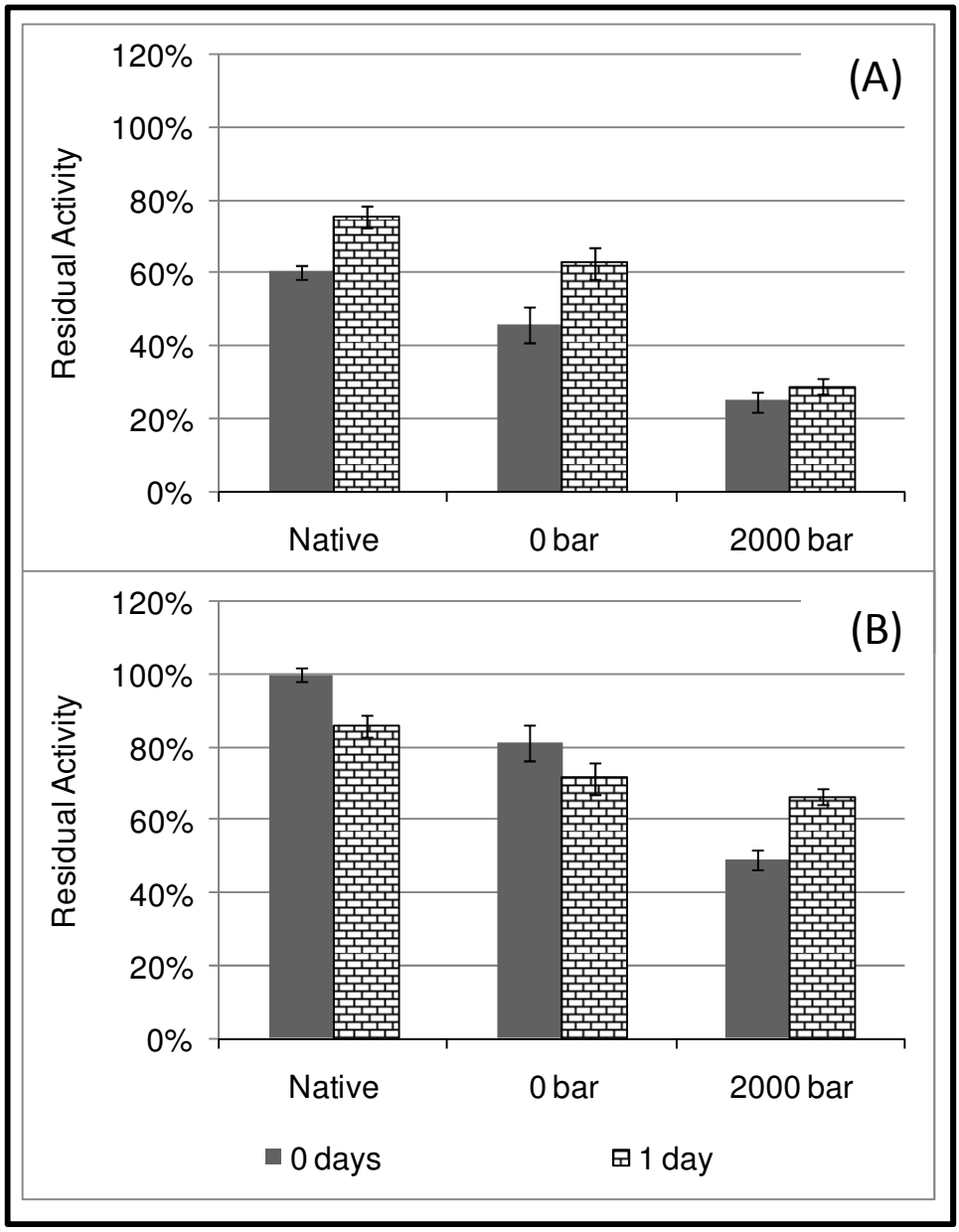

Figure 4.5. Effects of the HPH using inlet temperature of 60ºC on the protease activity measured at 20ºC (A) and 55ºC(B)

The results obtained for the native enzyme and for the pre heated native enzyme (*Figure 4.5*) showed no significant differences between them, indicating that the initial heating was not able to partially inactivate the enzyme. The activity of homogenized protease (0 bar) at a high inlet temperature showed a slight enzymatic activity reduction in all evaluated conditions. At 2000 bar, no differences were observed in the enzymatic activity at 55ºC, when compared with the non-heated enzyme (*Figure 4.2*). However, high differences were determined when the protease activity was measured at 20ºC, with an intense inactivation at high inlet temperature process. It probably indicates that the HPH at high temperatures (temperature after homogenization at 2000 bar = 64ºC) promoted a permanent change on enzyme configuration that abruptly reduced the activity at 20°C and was not recovered after one day of storage.

On contrary, this change seems to not affect the activity of protease at higher temperatures. Similar results were obtained by Welti-Chanes *et al*. (2009) after homogenization of orange juice at a high inlet temperature to inactivate pectinmethylesterase. These results demonstrated that the combination of homogenization and heating can be used in some cases, when enzyme inactivation is desirable. It is interesting to observe that the combination of a mild thermal process and a high pressure homogenization is also a promising method for the microorganisms inactivation (Tribst *et al.* 2011).

Considering the obtained results after storage at refrigerated temperature (that reduced the protease activity at 55°C) and those obtained after HPH at high inlet temperatures (that reduced the protease activity at 20°C), it is observed that when the protease was submitted to an extreme temperature conditions, its molecular conformation was permanently changed. Moreover, the new molecular conformation appears to be in accordance to the exposed temperature, reducing the enzyme activity at the opposite condition.

4.4. Conclusion

It was concluded that the HPH can promote reversible or irreversible changes in the *B. subtilis* neutral protease activity, promoting activation, inactivation and even changing enzyme optimum temperature. The obtained results highlight the HPH as an interesting tool to improve enzyme commercial applications.

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Capítulo 5. Increasing fungi amyloglucosidase activity by high pressure homogenization

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Resumo

A homogeneização à alta pressão (HAP) é um processo capaz de aumentar a atividade de algumas enzimas, portanto, o efeito da HAP sobre a amiloglicosidase (AMG) foi estudado. As soluções de enzima foram preparadas em pH 2,9, 4,3 e 6,5 e então processadas a pressão manométrica de 0 (amostras apenas circulando no equipamento), 500, 1000, 1500 e 2000 bar. O efeito foi determinado na atividade residual da enzima medida a 35, 65 e 80ºC. Os resultados obtidos a 35ºC não mostraram alterações significativas na atividade de AMG após a homogeneização a pressões de até 2000 bar para os três pH avaliados. De forma similar, a 65ºC (temperatura ótima) as enzimas nativas e homogeneizadas em pH 2,9 e 6,5 não mostraram alterações significativas. Por outro lado, quando a enzima foi homogeneizada em pH 4.3 e teve a atividade medida em temperatura ótima, foi observado um aumento significativo na atividade (5-8%) quando utilizadas pressões iguais ou superiores a 1000 bar. A 80ºC, a atividade aumentou após a homogeneização nos três pH avaliados, sendo que, em pH 2,9 a atividade apresentou um aumento gradual e significativo, atingindo um aumento máximo de 100% após homogeneização a 2000 bar. Em pH 4,3 e 6,5, a homogeneização em pressões iguais ou superiores a 1000 bar resultaram em um aumento significativo de atividade de 20 e 30%, respectivamente. Assim, os resultados indicaram que a HAP pode aumentar a atividade da amiloglicosidase, sendo dependente do pH da solução e das condições de homogeneização aplicadas. Além disso, foi observado que o processo pode modificar a atividade da AMG em diferentes temperaturas, sendo mais interessante quando é desejável a aplicação desta enzima em altas temperaturas.

Palavras-Chave: *amiloglicosidase • ultra alta pressão de homogeneização • atividade enzimática*

Abstract

High pressure homogenization (HPH) was recently described as a process able to improve the activity of some enzymes; therefore, the HPH effects on amyloglucosidase (AMG) were investigated. Enzyme solution at pH 2.9, 4.3 and 6.5 were processed at pressures of 0 (just sample circulation on the equipment), 500, 1000, 1500 and 2000 bar and the HPH effects were determined through the enzyme residual activity measured at 35, 65 and 80ºC. Results at 35ºC showed no relative changes on AMG activity after HPH up to 2000 bar for the three evaluated pH. Similarly, at 65ºC (optimum temperature), native and homogenized enzyme at pH 2.9 and 6.5 showed no significant activity changes. On the contrary, when the enzyme was homogenized at pH 4.3 and its activity evaluated at optimum temperature, a significant activity increase (5-8%) was observed after homogenization at pressures of 1000 bar and above. At 80ºC, it was observed an AMG relative activity increase after HPH for the three evaluated pH. Sample homogenized at pH 2.9 showed a gradual and significant activity increase, reaching a maximum increment of 100% after homogenization at 2000 bar with reference to the native enzyme. At pH 4.3 and 6.5, homogenization up to 1000 bar resulted on a significant AMG activity increase of around 20 and 30%, respectively. Therefore, the results highlighted that HPH can increase AMG activity, being dependent on the pH of enzyme solution and the applied pressure. Also, it was observed that the process can change the AMG activity at different temperatures, being especially interesting when AMG activity at high temperature is required.

Key-words: *amyloglucosidase • ultra-high pressure homogenization • enzymatic activity*

5.1. Introduction

Amyloglucosidase (AMG) or Glucoamylase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3) is an enzyme able to produce glucose from starch by removing successively glucose units from the non-reducing end of amylose or amylopectin molecules of starch mainly by hydrolysis of the L-1,4 glucosidic bound (Svensson, Pedersen, Svendsen, Sakai, Ottesen, 1982, Adeniran, Abiose, & Ogunsua, 2010). Structurally, AMG are classified in 6 distinct types and the predominant type contains three distinct regions and two functional globular domains (Kumar, & Satyanarayana, 2009).

The main application of AMG is the starch saccharification, process that is carried out after starch gelatinization at 105^{o}C and liquefaction by thermostable α-amylase (Mamo, & Gessesse, 1999). Saccharified starch is used directly in the food industry, or converted to ethanol of high quality to be used in perfumes or alcoholic beverages and also in textile and pharmaceutical applications (Zanin, & Moraes, 1998, Kumar, & Satyanarayana, 2009); therefore, the AMG is one of the most economically important industrial enzymes (Rami, Das, Satyanarayana, 2000). Additionally to these main applications, the use of AMG, is growing in juice industry, aiming to hydrolysis the starch naturally found in some beverages or found due to unripe fruit (commonly having high content of starch) be mixed to ripe ones (Ribeiro, Henrique, Oliveira, Macedo, & Fleuri 2010).

High pressure homogenization (HPH) in an emerging technology applied to food preservation with a minimum sensory and nutritional damage (Tribst, Franchi, de Massaguer, & Cristianini 2011, Franchi, Tribst, & Cristianini, 2011). This process was able to inactivate vegetative bacterial (Campos, & Cristianini, 2007; Tribst, Franchi, & Cristianini, 2008) yeasts and molds (Tahiri, Makhlouf, Paquin, & Fliss, 2006, Tribst, Franchi, Cristianini, & de Massaguer, 2009, Tribst, Franchi, de Massaguer, & Cristianini 2011). Moreover, some studies evaluated the HPH consequences in protein (Vannini et al., 2008) and polysaccharides (Lacroix, Fliss, & Makhlouf, 2005). On enzymes, the homogenization has been able to promote enzyme activation (Liu et

al., 2009 a,b; Liu et al., 2010, Tribst, Augusto, & Cristianini, 2012), inactivation (Lacroix, Fliss, & Makhlouf, 2005, Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009) or no change on the enzyme activity (Tribst, & Cristianini, 2012). Therefore, the HPH effects expected depends on the type of the enzymes and also the applied pressure level (Liu et al., 2009 a,b, Tribst, Augusto, & Cristianini, 2012, Tribst, & Cristianini, 2012). The aim of this research was to evaluate the effects of the HPH on the activity of AMG and its stability after storage.

5.2. Material and Methods

5.2.1. Enzyme

A commercial amyloglucosidase was evaluated (Prozyn Biosolutions®, São Paulo, Brazil, www.prozyn.com - batch number I – 368592910). The enzyme was presented as a yellow powder obtained as a product of *Aspergillus niger* fermentation (expected molecular weight of 70-90 kDa), with optimum pH at 4.4-6.0 and activity temperature ranging between 40 and 65°C, with optimum at 65°C.

5.2.2. Enzymatic Activity

The enzymatic activity was determined following the method described by Rami, Das, & Satyanarayana (2000) with few modifications: 500 µL of enzymatic solution (0.1 grams of dried enzyme diluted in one liter of 0.05 M acetate buffer at a pH of 4.3) was added to 4 mL of soluble starch (pro-analysis degree with purity of 99.6%) solution at 0.5% (w/v) (Synth, Brazil). The reaction was carried out at 65ºC for 10 minutes and stopped through addition of 3 mL of 1M Tris-HCl buffer at pH 7.5. The starch hydrolysis was determined through glucose release, measured using an enzymatic kit of Glucose Oxidase (Laborlab, Guarulhos, SP, Brazil), through a colorimetric reaction (Fleming, & Pegler, 1963). Samples absorbance were measured at 510 nm using UV-VIS spectrophotometer DU 800 (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme (U) was defined as the amount of enzyme able to produce

one µmol of glucose during reaction time, i.e. 10 minutes. Tubes containing only starch and only enzyme were used as controls.

The standard curve was obtained using 10, 8, 6, 4, 2, 1, 0.5, 0.25 and 0.125 mmol of glucose solution. The glucose reacted with enzymatic kit of Glucose Oxidase and after samples absorbance was measured at 510 nm in triplicate.

5.2.3. Optimum pH and temperature

The AMG activity was carried out at pH 2.2, 2.9, 3.8, 4.3, 5.0, 5.7 and 6.5 using 0.05 M of citrate-phosphate, acetate or phosphate buffer. The effect of the temperature incubation was evaluated at 35, 50, 65 and 80ºC. The enzymatic activity was measured by starch hydrolysis method (previously described), changing the pH and the temperature of enzymatic reaction. The condition that presented higher activity (pH and temperature) was established as optimum with 100% of enzymatic activity. The residual activity was calculated using the *Equation 5.1*.

$$\text{Residual activity (\%)} = (\text{activity}_{\text{non\_optimum\_pH\_and\_temperature}} / \text{activity}_{\text{optimum\_pH\_and\_temperature}}) .100$$

(*Equation 5.1*)

5.2.4. High Pressure Homogenization of Amyloglucosidase at Room Inlet Temperature

A High-Pressure Homogenizator Panda Plus (GEA-Niro-Soavi, Parma, Italy) was used in the tests. The equipment contains a single acting intensifier pump that amplifies the hydraulic pressure up to 2000 bar.

A volume of 2 L of the AMG solution at 26.7ºC (pH 2.9, 4.3 and 6.5) was homogenized under pressures of 0 (obtained by pumping the enzyme solution through the homogenizer with no pressure applied), 500, 1000, 1500 and 2000 bar. The 0 bar was evaluated since the small gaps on the equipment (even when no

pressure is applied) can change some molecules structures, as showed for protease (Tribst, Augusto, & Cristianini, 2012).

Samples (50 mL) were collected and unprocessed AMG (native) was evaluated as a control, as previously described by Tribst, & Cristianini (2012).

Enzyme activities were performed at 35ºC, 65ºC, and 80ºC and measured through starch hydrolysis method. The assays were performed just after HPH and after 24 h of refrigerated storage for native and AMG homogenized at 1000 and 2000 bar.

5.2.5. High Pressure Homogenization of Amyloglucosidase at High Inlet Temperature

A sample of AMG solution (pH 2.9, 4.3 and 6.5) was homogenized (2000 bar) at inlet temperature of 65ºC, using the same procedure described for enzyme at room temperature. The 65ºC was chosen since it is almost the optimum temperature of the enzyme, aiming to evaluate if the effects of HPH on enzyme improve at this condition. The enzymatic activity was performed at 35ºC, 65ºC, and 80ºC, just after homogenization and after 24h of refrigerated storage.

5.2.7. Statistical Analysis

The analysis of variance (ANOVA) was performed to compare the effects of the different treatments and the Tukey test was used to determine the difference of them at a 5% confidence level. Statistical analyses were carried out in STATISTICA 5.0 software (StatiSoft, Inc., Tulsa, Okla., U.S.A.). All of the tested conditions and determinations of the AMG activity were triplicated. The results were represented as mean ± standard deviation.

5.3. Results and Discussion

5.3.1. Optimum pH and temperature

The AMG activity at different pH and temperatures is shown in *Figure 5.1*. The absorbance were converted into glucose concentration using a glucose standard curve determined through linear regression of the glucose absorbance data ([glucose_concentration] =10233.abs_{510nm} - 226, with R^2=0.999).

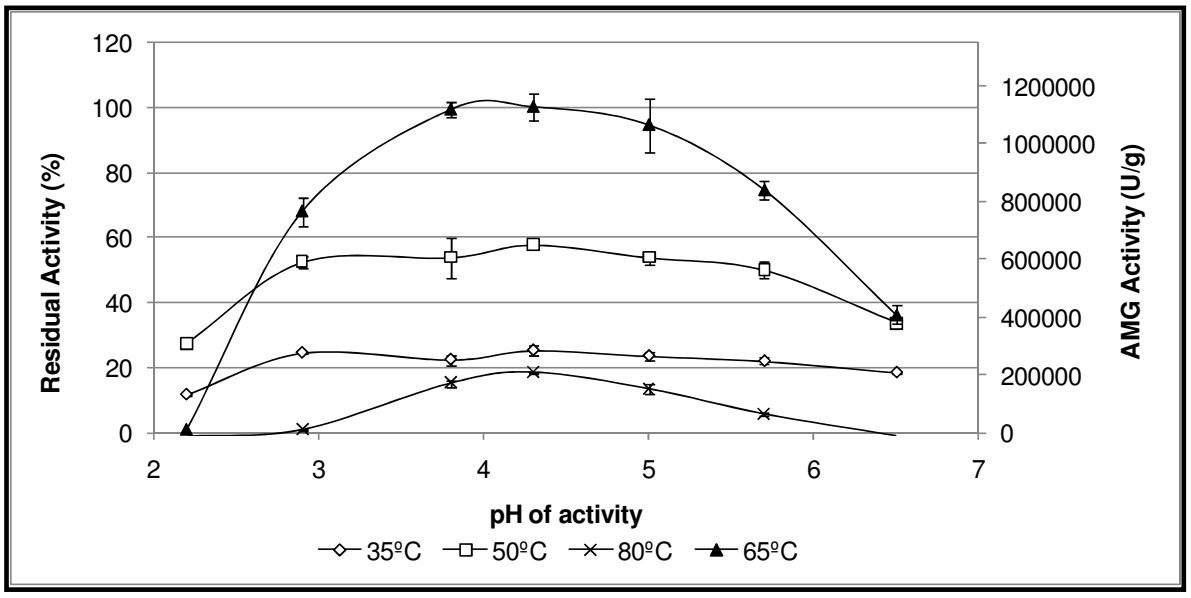

Figure 5.1. Effect of pH (2.2-6.5) and temperature (35 - 80ºC) on AMG activity

The optimum AMG conditions, i.e., the condition at a higher activity, were determined as a pH 4.3 and a temperature of 65ºC. At this condition the activity was 1,126,575 U per gram of dried enzyme, which was considered as 100% of residual activity. The enzyme was highly stable between pH 2.9 and 5.8, with residual activity higher than 60% in this range. On contrary, pH 2.2 was able to denature the enzyme when associated with temperatures above 65ºC, being negligible the activity at these conditions. The temperature variation resulted in significant changes on enzymatic activity, reducing around 40% of residual activity at 50ºC and 80% at 35 and 80ºC. An

improvement on pH stability was observed at temperatures under 65ºC, indicating that elevated temperature associated with low or high pH potentiates the AMG activity loss.

Considering the results showed in *Figure 5.1*, the measurement of AMG activity was evaluated at pH of 4.3 after HPH (optimum pH). The temperatures of 35, 65 and 80º C were studied to evaluate if the high pressure homogenization affected the enzyme activity in non-ideal conditions, since previous results indicated that HPH is able to improve enzyme activity at non-optimum temperatures (Tribst, Augusto, & Cristianini, 2012) and also enzyme stability at high temperature (Liu et al., 2010). Considering the main use of AMG on starch saccharification – process where starch need to be previously heated at higher temperatures (above 80ºC) to be gelatinized (Mamo, & Gessesse, 1999) –, the activity of AMG at temperatures higher than 65ºC is desirable, allowing the enzyme application immediately after saccharification, resulting in time and energy economy.

5.3.2. High Pressure Homogenization of Amyloglucosidase at Room Inlet Temperature

The HPH processes were carried out at pressures up to 2000 bar. The fast decompression during homogenization promotes intense shear and friction with consequent heating of the homogenized fluid. Considering that enzymes can be affected by heating, the sample temperature reached at each pressure was measured. The residence time at those temperatures was in the order of 10 s. *Figure 5.2* shows the temperatures reached after homogenization.

The pressure increase resulted in a linear temperature increment in the enzyme solution of around 1.2 ºC for each 100 bar; in addition it was observed that the maximum temperature was 52.1ºC at 2000 bar. This temperature was not enough to promote enzyme thermal denaturation (temperature lower than optimum temperature of AMG activity) and, consequently, all the effects observed after the HPH can be only attributed to the homogenization process. The effects of the HPH on AMG activity measured at different temperatures are shown in *Figure 5.3.*

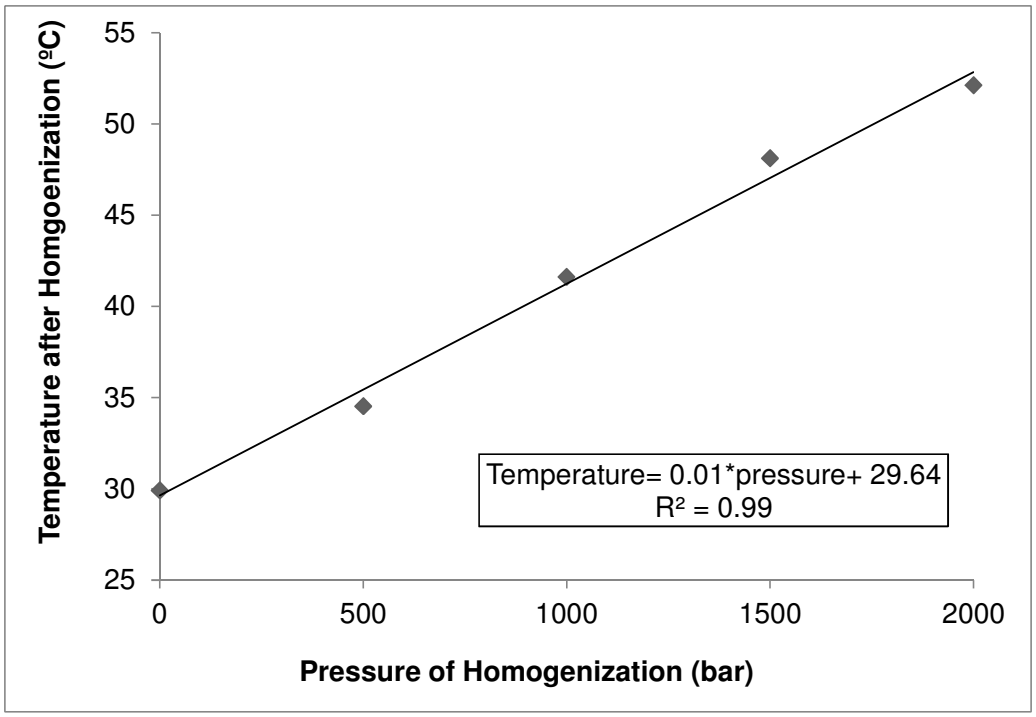

Figure 5.2. Temperature increase during HPH (inlet temperature = 26.7ºC)

Results showed no statistical differences between the triplicate of each evaluated sample, indicating good repeatability of process and analysis methodology. The native enzyme activity was affected by the pH of homogenization and the temperature of activity measurement. Therefore, the effect of HPH on the AMG was evaluated for each temperature and pH, comparing the results of native and homogenized samples.

Results at 35ºC showed no relative changes on AMG activity after HPH up to 2000 bar for the three evaluated pH ($p > 0.05$). Similarly, at 65ºC (optimum temperature), native and homogenized enzyme at pH 2.9 and 6.5 showed no relative changes on AMG activity ($p > 0.05$). On contrary, when enzyme was homogenized at pH 4.3 and its activity measured at optimum temperature, a relative activity increase (5-8%) was observed after homogenization up to 1000 bar ($p < 0.05$).

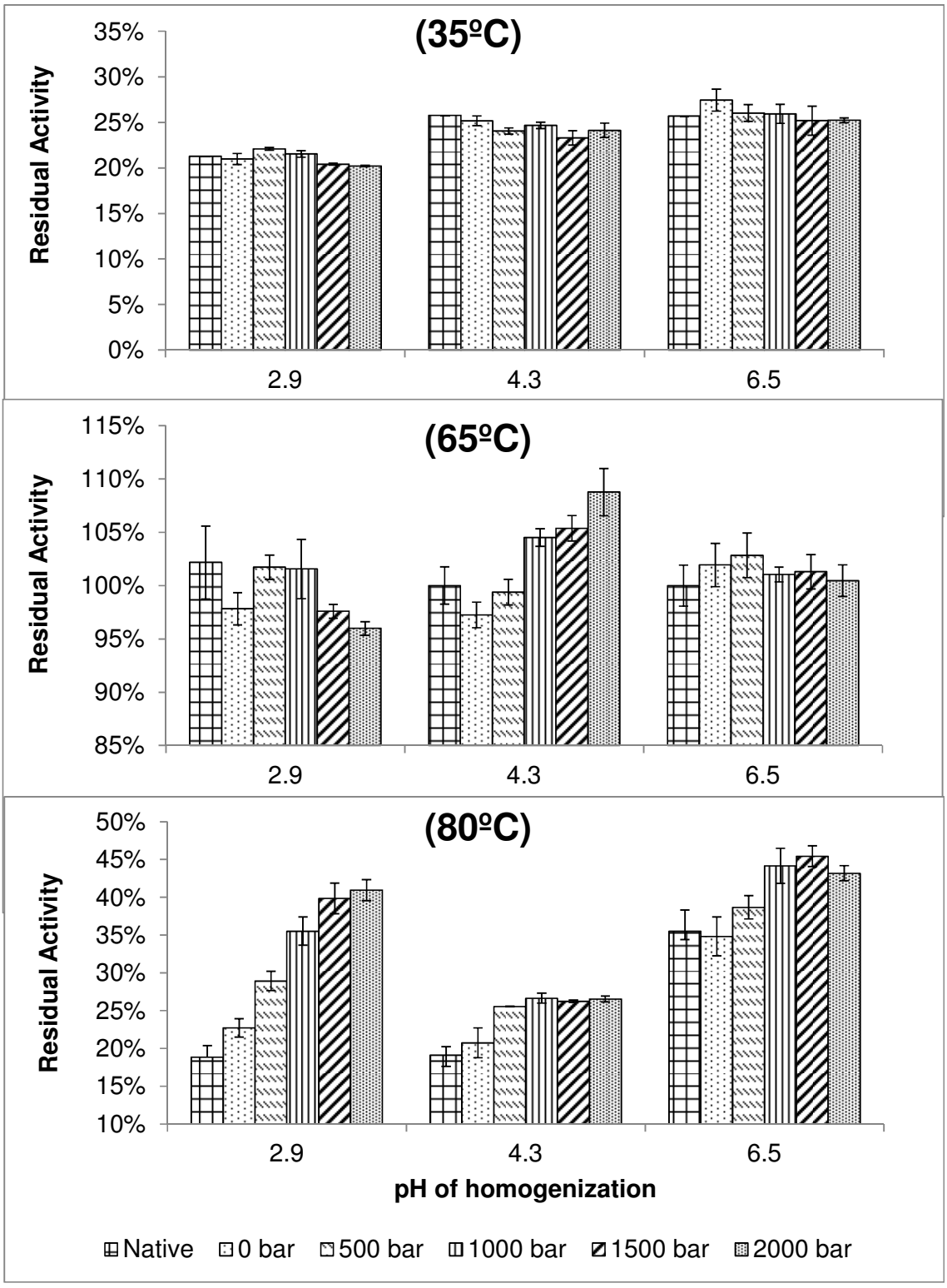

Figure 5.3. Effects of HPH between 0 and 2000 bar on the AMG activity measured at 35, 65, and 80ºC

At 80ºC, it was observed an AMG relative activity increase after HPH for the three evaluated pH ($p< 0.05$). Sample homogenized at pH 2.9 showed a gradual and significant relative activity increase, reaching a maximum increment of 100% after homogenization at 2000 bar. At pH 4.3 and 6.5, homogenization up to 1000 bar resulted on AMG relative activity increase of around 20 and 30% ($p<0.05$), respectively. Therefore, the results highlighted that HPH can improve AMG relative activity, being dependent on the pH of enzyme solution and the applied pressure. Also, it was observed that process can change the AMG activity at different temperatures, which can be especially interesting when AMG is applied to starch saccharification (Mamo, & Gessesse, 1999), since it allows the enzymatic process be carried out at higher temperatures than optimum, with energy and time economy (Mamo, & Gessesse, 1999).

Previous results indicate that HPH was able to improve (Liu et al. 2009 a,b, Liu et al. 2010, Tribst, Augusto, & Cristianini, 2012), reduce (Lacroix, Fliss, & Makhlouf, 2005, Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009) or cause no change (Tribst, & Cristianini, 2012) on the activity of enzymes at pressures in the same range studied in the present work, being the effects specific for each enzyme and the level of applied pressure (Liu et al. 2009a,b). Tribst, & Cristianini (2012) evaluated the effects of HPH on commercial α-amylase and observed no changes on enzyme activity after process up to 1500 bar, highlighting that different effects of HPH can be observed even for enzymes of the same subclass.

5.3.3. Storage effect at 8°C for 24 hours on activity of AMG

The AMG relative activity was measured after one day of storage at 8ºC, aiming to evaluate the enzyme stability at this condition. The native and homogenized enzyme at 1000 and 2000 bar were studied to evaluate if the enzyme changes caused by intermediate and maximum HPH are transitory or permanent, and also if HPH was able to change the enzyme stability in aqueous solution at different pH. The results are shown in *Table 5.1.*

Table 5.1. Residual AMG activity at 35, 65 and 80ºC after one day of storage (8ºC) at pH 2.9, 4.3 and 6.5

Temperature of activity	Sample	pH 2.9		pH 4.3		pH 6.5	
		0 day	1 day	0 day	1 day	0 day	1 day
35ºC	Native	21.3 ± 0.0%^{a*}	22.8 ± 0.4%^{a,b}	25.8 ± 0.0%^{a}	19.9 ± 1.7%^{b}	22.3 ± 0.0%^{a}	24.3 ± 1.7%^{a}
	1000 bar	21.5 ± 0.4%^{a}	26.6 ± 1.6%^{c}	24.7 ± 0.4%^{a}	19.0 ± 0.5%^{b}	22.5 ± 0.9%^{a}	25.7 ± 1.0%^{a}
	2000 bar	20.2 ± 0.1%^{a}	24.9 ± 1.1%^{b,c}	24.1 ± 0.8%^{a}	18.5 ± 0.3%^{b}	21.9 ± 0.2%^{a}	24.4 ± 0.8%^{a}
65ºC	Native	100.8 ± 6.2%^{a}	98.9 ± 3.1%^{a}	100.0 ± 1.8%^{a}	102.0 ± 0.7%^{a}	100.0 ± 1.9%^{a}	100.8 ± 5.4%^{a}
	1000 bar	101.6 ± 2.8%^{a}	97.6 ± 1.9%^{a}	104.5 ± 0.9%$^{a.b}$	99.1 ± 3.4%^{a}	101.0 ± 0.7%^{a}	103.6 ± 3.3%^{a}
	2000 bar	96.0 ± 0.6%^{a}	95.9 ± 3.6%^{a}	108.8 ± 2.3%^{b}	89.1 ± 1.6%^{c}	100.5 ± 0.0%^{a}	103.3 ± 1.2%^{a}
80ºC	Native	18.8 ± 1.5%^{a}	5.5 ± 1.5%^{d}	19.1 ± 1.1%^{a}	20.8 ± 1.6%^{a}	30.9 ± 2.4%^{a}	10.3 ± 0.6%^{b}
	1000 bar	35.5 ± 1.9%^{b}	8.3 ± 0.5%^{d}	26.7 ± 0.7%^{b}	19.2 ± 0.3%^{a}	38.4 ± 2.0%^{c}	10.6 ± 0,8%^{b}
	2000 bar	41.0 ± 1.4%^{c}	13.0 ± 1.3%^{e}	26.5 ± 0.4%^{b}	18.5 ± 0.9%^{a}	37.5 ± 0.8%^{c}	13.3 ± 1.0%^{b}

***** Different letters means significant differences on results ($p < 0.05$), data was evaluated individually for each temperature of activity

Native enzyme activity was affected by the pH of storage solution and also by the temperature of activity measurement; therefore, again, results were evaluated for each temperature and pH, comparing the results of native and homogenized samples. The native sample activity just after preparation and after one day of storage showed no differences when activity was measured at 35 and 65ºC for all evaluated pH, showing that AMG was stable after solution preparation, being similar to the results previously observed for α-amylase (Tribst, & Cristianini, 2012). When activity was measured at 80ºC, on contrary, a significant reduction was observed for AMG samples stored at pH 2.9 and 6.5, showing that storage at non-optimum pH affected the activity of native enzyme at high temperature.

The pH of solution, the pressure applied and the temperature of activity measurement affected the AMG stability. However, just when the enzyme was homogenized, stored at pH 2.9 and relative activity was measured at 35ºC the activity after one day of storage was higher than the activity of the native one stored at the same condition, showing that homogenization did not improve the enzyme storage stability for all other evaluated condition.

The relative activity improvement at 65ºC (just sample homogenized at pH 4.3) and 80ºC observed just after homogenization was not permanent, since the results of relative activity after one day of storage showed that homogenized samples presented activity equal or lower than the native one after the same period. Therefore, although HPH was able to improve the enzyme activity in some conditions, probably due to spatial configuration changes and exposure of active sites (Liu et al., 2009b, Tribst, Augusto, & Cristianini, 2012), these changes were reversible for AMG. In fact, the equal activity for native and homogenized samples in some conditions indicates that homogenized AMG returned to its native configuration after a rest period.

5.3.4. High Pressure Homogenization of Amyloglucosidase at High Inlet Temperature

The effect of homogenization at high temperature was evaluated, carrying out the homogenization process at inlet temperature of 65ºC, which is the optimum temperature for enzyme activity. The temperature monitoring during processing showed that the maximum temperature reached was 70.3º C at 1500 bar. Although the reached temperature was higher than the optimum temperature of AMG, an enzyme thermal inactivation was not expected, mainly due the small residence time at high temperature (< 10s). The results are shown in *Table 5.2*.

Results showed that enzyme preparation at high temperature was enough to promotes relative activity loss, mainly when pH of solution was non-optimum (2.9 and 6.5), indicating that association of non-optimum pH and high temperature promotes AMG inactivation.

Table 5.2. Residual AMG activity after homogenization at high inlet temperature (65ºC)

Temperature of activity	pH*	Sample			
		Native at 20ºC	Homogenized at 20ºC of inlet	Native at 65ºC	Homogenized at 65ºC of inlet
35ºC	2.9	21.3 ± 0.0%^{a**}	20.2 ± 0.1%^{a}	17.0 ± 0.0%^{b}	0.1 ± 0.2%^{c}
	4.3	25.8 ± 0.0%^{a}	24.1 ± 0.8%^{a}	19.6 ± 0.0%^{b}	1.0 ± 0.6%^{c}
	6.5	22.3 ± 0.0%^{a}	21.9 ± 0.2%^{a}	1.5 ± 0.0%^{b}	0.3 ± 0.2%^{b}
65ºC	2.9	100.8 ± 6.2%^{a}	96.0 ± 0.6%^{a,b}	93.5 ± 4.2%^{b}	0.0 ± 0.1%^{c}
	4.3	100.0 ± 1.8%^{a}	108.8 ± 2.3%^{b}	98.0 ± 0.0%^{a}	8.7 ± 1.6%^{b}
	6.5	100.0 ± 1.9%^{a}	100.5 ± 0.0%^{b}	12.4 ± 0.0%^{c}	4.5 ± 0.6%^{d}
80ºC	2.9	18.8 ± 1.5%^{a}	41.0 ± 1.4%^{b}	12.0 ± 0.0%^{a}	1.7 ± 3.5%^{c}
	4.3	19.1 ± 1.1%^{a}	26.5 ± 0.4%^{b}	15.7 ± 0.0%^{a}	1.6 ± 0.1%^{c}
	6.5	30.9 ± 2.4%^{a}	37.5 ± 0.8%^{b}	2.4 ± 0.0%^{c}	1.2 ± 0.1%^{c}

* pH of homogenization ** Different letters means significant differences on results ($p < 0.05$), data was evaluated individually for each temperature of activity

The homogenization at high inlet temperature was highly deleterious for AMG activity at all evaluated conditions, with activity loss higher than 90%. Considering that reached temperature during the process and HPH at 2000 bar (*Figure 5.2*) were individually not able to promote this level of enzyme inactivation, it was concluded that homogenization associated to temperature had a synergistic effect on AMG inactivation. Also, the activity evaluation after one day of storage at 8ºC showed that this inactivation was not reversible, since the relative activity at 65ºC of sample homogenized and stored at pH 4.3 was 9.4±1.3%, with no significant difference with sample activity just after homogenization. Therefore, it can be concluded that homogenization at 65ºC was deleterious for the enzyme activity. In contrast, the results highlighted that HPH of AMG at high inlet temperatures can be a very interesting way to inactivate the enzyme at the end of the hydrolysis process without using thermal processing.

5.4. Conclusion

High pressure homogenization was able to relatively mantain or increases the amyloglucosidase activity immediately after homogenization, depending on the pH of homogenization and the temperature of activity. Best results were obtained at 80ºC, which is very interesting especially when AMG is applied in starch saccharification process, which requires enzyme active at higher temperatures, for improving time and energy economy.

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Capítulo 6. The effect of high pressure homogenization on the activity of a commercial β-Galactosidase

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Resumo

A homogeneização à alta pressão (HAP) tem sido proposta como um método promissor para modificar a atividade e a estabilidade de enzimas. Assim, este trabalho estudou a atividade de uma β-galactosidase comercial antes e após a HAP. Uma solução de enzima foi preparada em pH 6,4, 7,0 e 8,0 e processada em pressões de homogeneização de até 150 MPa. A atividade residual da enzima foi medida a 5, 30 e 45ºC imediatamente após a homogeneização e após um dia de estocagem refrigerada a 8ºC. Os resultados demonstraram que a enzima permaneceu ativa a 30 ºC (temperatura ótima) em pH neutro mesmo após HAP de até 150 MPa. Por outro lado, quando a β-galactosidase foi homogeneizada a pH 6,4 e 8,0, observou-se uma perda gradual de atividade, atingindo um mínimo de 30% após homogeneização a 150 MPa e pH 8,0.

Após a estocagem, apenas a β-galactosidase homogeneizada em pH 7,0 permaneceu com atividade similar a amostra nativa. Portanto, a HAP não afetou a atividade e a estabilidade da β-galactosidase apenas quando o processo foi realizado em pH neutro e, para as demais condições avaliadas, a homogeneização à alta pressão resultou em inativação parcial da enzima. Considerando que a β-galactosidase é utilizada para produzir leite delactosado, pode-se concluir que a homogeneização à alta pressão pode ser aplicada no leite adicionado da enzima, sem promover a inativação da enzima.

Palavras-Chave: *ultra alta pressão de homogeneização • atividade enzimática • processo não térmico • leite com baixo teor de lactose*

Abstract

High pressure homogenization (HPH) has been proposed as a promising method for changing the activity and stability of enzymes. Therefore, this research studied the activity of β-galactosidase before and after HPH. The enzyme solution at pH values of 6.4, 7.0 and 8.0 was processed at pressures of up to 150 MPa, and the HPH effects were determined from the residual enzyme activity measured at 5, 30 and 45ºC immediately after homogenization and after one day of refrigerated storage. The results indicated that at neutral pH the enzyme remained active at 30 ºC (optimum temperature) even after homogenization at pressures of up to 150 MPa. To the contrary, when the β-galactosidase was homogenized at pH 6.4 and 8.0, a gradual loss of activity was observed, reaching a minimum activity (around 30%) after HPH at 150 MPa and pH 8.0. After storage, only β-galactosidase high pressure homogenized at pH 7.0 remained with similar activity of native sample. Thus HPH did not affect the activity and stability of β-galactosidase only when process was carried out at neutral pH; for the other conditions, HPH resulted in enzyme partial inactivation. Considering the use of β-galactosidase to produce low lactose milk, it was concluded that HPH can be applied with no deleterious effects on enzyme activity.

Keywords: *ultra high pressure homogenization – enzyme activity – non thermal technology – low lactose milk*

6.1. Introduction

High pressure homogenization (HPH) is an emerging technology developed to process food, aiming to minimize sensory and nutritional damages [7] when compared to the traditional thermal process. HPH is based on the homogenization process widely used in the dairy industry for breaking up fat globules [23], but applying pressures 10 times higher. Using high pressures, this process inactivates vegetative bacterial [4,23,28] yeast and molds cells [1,29,30]. Thus, HPH was proposed to improve the safety and microbiological quality of milk, being similar to pasteurization [9,10].

The effects of HPH on milk are not limited to microbial reduction and also include changes in the milk constituents, such as modifications in the ratio of the nitrogen fractions [11], changes in the soluble forms of calcium and phosphate [11], aggregation of whey proteins with the casein [6], fat globule size reduction [10] and greater dissolution of the α- and κ-caseins [26]. These physical changes improve the sensory characteristics of HPH milk, such as its mouth feel and aeration capacity [2]. Also, HPH is of interest to prepare milk for the manufacture of fermented dairy products owing to: (1) enhancement of proteolytic and lipolytic activity during cheese ripening [9], (2) maximization of starter growth during fermentation and also reduction of losses in viability during refrigerated storage [16,22], (3) enhancement of hydrophobic peptides during fermentation, which have potential biological activities [27], (4) reduction of syneresis and increase in firmness of fermented milk [22,27], (5) improvement of the aromatic profile of the fermented products [22], (6) improvement of the water binding capacity of cheese proteins with less whey separation [9].

Milk and dairy products are recognized as good sources of high-quality protein and calcium [5,14]. However, lactose intolerance, which affects 3-70% of people from different population groups, limits the digestion of these foods [12]. Moreover, lactose reduction improves the technological and sensory properties of dairy products [8,12,25]. Therefore, the production of lactose free or low lactose dairy products is desirable [12] and these could be obtained by the addition of β-galactosidase (EC

3.2.1.23) to the milk, because this enzyme catalyze the hydrolysis of lactose into glucose and galactose [12,13,19].

HPH was previously described as a process capable [15, 17,18,19,31, 32, 34] or otherwise [33] of changing enzyme activity and stability, the effects normally being associated with the individual enzyme being evaluated and with the homogenization pressure applied [17,18].

Data about pectin methyl esterase indicated that homogenization was just able to inactivate the enzyme [15,34], whereas results obtained for polyphenol oxidase showed that HPH between 120 and 160 MPa causes an increase of enzyme activity [17,18]. Data obtained for α-amylase [33] and trypsin [19] showed no changes on enzyme activity upon HPH; however, an increase on trypsin thermal stability was observed [19]. Data on neutral protease [32] and amyloglucosidase [31] revealed that these enzymes can be activated or inactivated depending on the homogenization pressure applied, pH of enzyme solution and the temperature of activity measurement [31,32]. When passing through the homogenizer, the sample is submitted to a shear stress whose mechanical energy results in conformational change of enzyme molecule. When an enzyme undergoes conformational changes, either activation or inactivation may be expected. Conformational change may expose the active site and increase its activity, or it may prevent its contact with the substrate, thus reducing enzyme activity. It is therefore not possible to establish a fixed rule about the homogenization effects on enzymes.

The effect of HPH on β-galactosidase has not yet been evaluated. Considering the growing importance of the HPH process in the production of dairy products, the objective of this work was to evaluate the stability of this enzyme to HPH processing.

6.2. Material and Methods

6.2.1. β-Galactosidase and enzyme activity

The β-galactosidase used in these experiments was a commercial enzyme from Prozyn Biosolutions® (São Paulo, Brazil; batch number 368592910). The enzyme is a yellow, viscous liquid obtained as a fermentation product from

Kluyveromyces lactis. It is a dimeric enzyme composed of two identical subunits, with an expected molecular weight of 200 kDa.

The enzyme was evaluated at a concentration of 0.1% (w/v) and the enzyme activity was determined following the method previously described [12] with a few modifications. The enzyme solution was prepared in a 0.1 M phosphate buffer at pH 7.0, and 300 µL of this solution added to 3 mL of a 2% lactose solution (w/v) (Synth, Brazil). The reaction was carried out at 30ºC for 15 minutes and stopped by immersing the tubes in boiling water for 5 minutes. The tubes were then cooled in an ice bath.

Lactose hydrolysis was determined from the release of glucose, as measured using a Glucose Oxidase enzyme kit (Laborlab, Guarulhos, SP, Brazil), involving a colorimetric reaction. Sample absorbance was measured at 510 nm using a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme was defined as the amount of enzyme able to produce one µmol of glucose per minute of reaction and per gram of enzyme. Tubes containing only lactose and only enzyme were used as the controls. The galactose and lactose present in the medium had no influence on the glucose determination.

6.2.2. Optimum pH and temperature

β-galactosidase activity was evaluated at pH values of 5.7, 6.4, 7.0, 7.5, and 8.0. The assays were carried out in 0.1 M acetate buffer (pH 5.7) and 0.1 M phosphate buffer (pH 6.4-8.0). The effect of temperature on enzyme activity was evaluated at 4, 15, 30, 45, 60 and 75ºC. β-galactosidase activity was determined by the glucose oxidase method, modifying the pH and incubation temperature. A high concentration of β-galactosidase (0.5%) was evaluated, aiming to determine the activity even under extreme conditions. The conditions for maximum activity (pH and temperature) were considered as the optimum conditions, denominated as 100% of enzymatic activity. The residual activity was calculated according to *Equation 6.1*.

$$\text{Residual activity (\%)} = (\text{Activity under non ideal conditions} / \text{optimum activity}) \cdot 100 \qquad (\textit{Eq. 6.1})$$

6.2.3. High pressure homogenization of β-galactosidase at an inlet temperature of 8.5 ºC

A Panda Plus High-Pressure Homogenizer was used (GEA-Niro-Soavi, Parma, Italy). The equipment contains a single acting intensifier pump that amplifies the hydraulic pressure up to 150 MPa. The pressure at the second stage valve was set at 0 MPa (gauge pressure). The equipment flow rate is 9 $L \cdot h^{-1}$. Two liters of the β-galactosidase solution at 8.5ºC (pH 6.4, 7.0 and 8.0) were homogenized at pressures of 0 (obtained by pumping the enzyme solution through the homogenizer with no pressure applied), 50, 100 and 150 MPa. Samples (50 mL) were collected as previously described [31,32,33], and non-processed β-galactosidase (native) solution was used as the control sample.

Enzyme activity was determined at 5, 30 and 45ºC using the glucose oxidase method. The UV absorption spectrum of the enzyme was obtained and evaluated following the method described elsewhere [32]. Both assays were performed immediately after HPH and after 24h of refrigerated storage for the native β-galactosidase and that homogenized at 50 and 150 MPa.

6.2.4. High pressure homogenization of β-galactosidase with an inlet temperature of 20 ºC

HPH of the β-galactosidase solution at pH 7.0 was carried out with an inlet temperature of 20ºC, using the same procedure described for the enzyme at 8.5ºC. As previously described [31,32], the HPH process promotes intense shear and friction and involves the dissipation of mechanical energy as thermal energy, increasing the product temperature [24].Therefore the highest inlet temperature was set at 20ºC, bacause higher temperatures would thermally denature the enzyme during processing. The enzyme activity was determined at 5, 30 and 45ºC, immediately after homogenization and after one day of storage.

6.2.5. Statistical analysis

The analysis of variance (ANOVA) was carried out to compare the effects of the different treatments, and the Tukey test was used to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 5.0 software – (StatiSoft, Inc., Tulsa, Okla., U.S.A.). All the processes and the determination of β-galactosidase activity were carried out in triplicate. The results were represented as the mean ± standard deviation.

6.3. Results and discussion

6.3.1. Enzyme characterization

The effects of pH and temperature on β-galactosidase activity are shown in *Figure 6.1*. The optimum conditions for enzyme activity were determined as pH 7.0 and 30ºC. Under these conditions the activity was 60,894 U/g, which was considered as 100% of residual activity. The variation in pH resulted in significant loss of enzyme activity, reducing the activity by up to 50% at pH 6.4 and 8.0. Variation in the temperature also affected β-galactosidase activity, with a reduction of around 40% and 80% at 15 and 45ºC, respectively. The results also demonstrated that the enzyme had low thermal stability, being completely inactivated at 60ºC. On the other hand, it remained active at 5ºC (around 20% of residual activity), which could be useful when the milk is stored cold for a period before processing. The optimum conditions observed (neutral pH and low temperature) were to be expected for the β -galactosidase produced by yeasts [8,12,13].

Considering the results shown in *Figure 6.1*, pH 7.0 and 30°C were chosen as the ideal conditions to measure the activity of β-galactosidase before and after HPH.

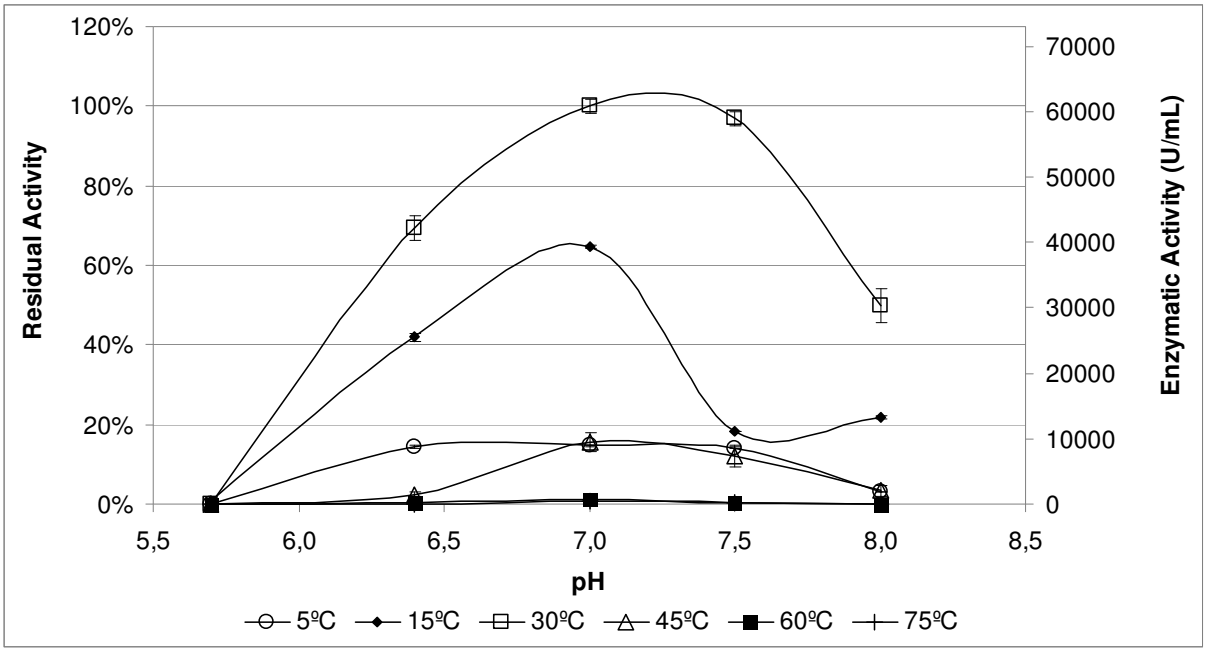

Figure 6.1. Effect of pH (6.4, 7.0 and 8.0) and temperature (5, 30 and 45ºC) on β-galactosidase activity

6.3.2. High pressure homogenization of β-galactosidase with an inlet temperature of 8.5 ºC

The HPH processes were carried out at pressures of up to 150 MPa. The fast decompression during HPH promotes intense shear and friction, with consequent heating of the product. Since enzymes can be affected by heating, the temperature reached under each set of process conditions was also evaluated. The residence time at those temperatures was ± 10s, and *Table 6.1* shows the temperatures reached after homogenization.

The increase in pressure promoted a linear increase in temperature of the enzyme solution of around 1.3ºC/ 10 MPa, the maximum temperature (32.6º C) being reached at 150 MPa. This temperature was too low to promote thermal denaturation of the enzyme during the process residence time, and consequently, all the effects observed after HPH can be attributed exclusively to the homogenization process.

Table 6.1. Increase in temperature during HPH (inlet temperature = 8.5ºC)

Pressure (MPa)	Final Temperature (ºC)	Temperature Increment (ºC)
0	13.0	4.5
50	18.1	9.6
100	25.6	17.1
150	32.6	24.1

The enzyme changes due to HPH were evaluated by UV-absorption spectra analysis of the homogenized β-galactosidase at pH 6.4, 7.0 and 8.0, at 8.5° C. The results for UV-absorption are shown in *Figure 6.2.*

The results showed that the pH of the solution significantly affected the UV-absorption peaks, the enzyme peak being lower at pH 6.4 than at pH 7.0. At pH 8.0, no statistical differences were observed between the peaks at pH 6.4 and 7.0. Therefore to determine the effects of HPH, each pH value was evaluated separately.

At pH 6.4, significant differences were observed between the native enzyme and the sample homogenized at 150 MPa. At pH 8.0, all the homogenized samples (50, 100 and 150 MPa) were different from the native sample. On the contrary, at pH 7.0, no significant differences were observed between the native and homogenized samples. This demonstrates that homogenization at different pH values promotes specific changes in the enzyme, and that the enzyme was more stable at its optimum pH value. Also, the differences observed showed a tendency for the UV-absorption of the enzyme to increase after homogenization.

The increase in UV-absorption was associated with the gradual exposure of tyrosine and tryptophan hydrophobic residues after HPH processing [18,19]. Therefore, the results obtained indicated that the hydrophobic residues of β-galactosidase were stable to HPH at pH 7.0 and highly unstable at pH 8.0.

The reversibility of the HPH changes was evaluated from the enzyme UV-absorption peak after one day of storage at 8ºC at the different pH values (*Figure 6.2*). The peaks measured immediately after homogenization and after one day of storage were different for all the conditions of pH and homogenization, indicating that the enzyme changes its configuration after one day in a buffer solution.

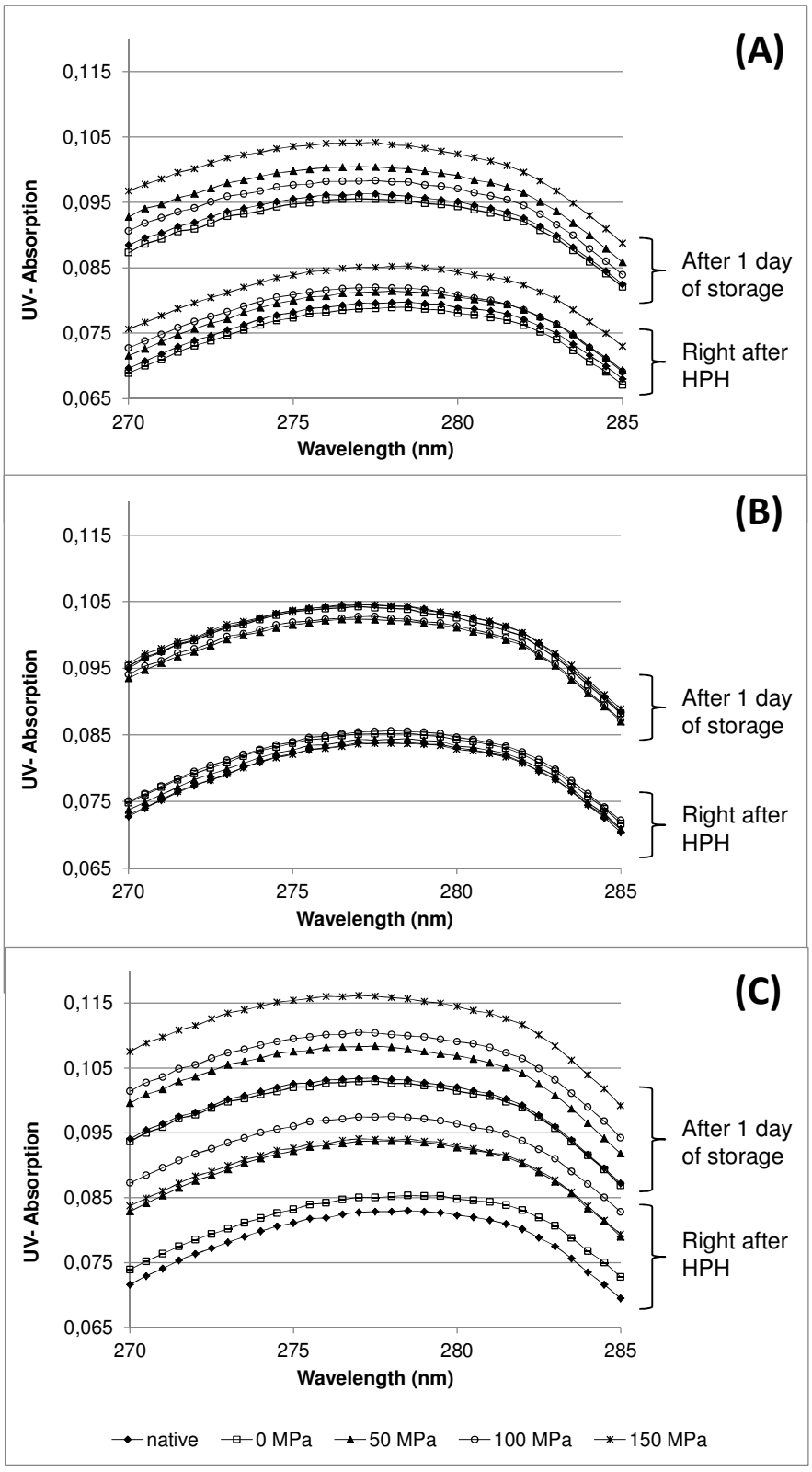

Figure 6.2. UV-absorption spectra of β-galactosidase at pH 6.4 (A), pH 7.0 (B) and pH 8.0 (C), immediately after homogenization and after one day of rest at 8º C

Comparing the results for the native and HPH enzymes after the storage period, no differences were observed between samples at pH 7.0. At pH 6.4 and pH 8.0, only the enzymes homogenized at 150 MPa were different from the native sample. This may indicate that the changes caused by homogenization at 50 and 100 MPa at a pH value of 8.0 were reversible, while the changes occurring at 150 MPa at pH values of 6.4 and 8.0 appeared to be permanent.

Figure 6.3 shows the results for the effects of HPH on β-galactosidase activity at 5, 30 and 45ºC, measured immediately after homogenization at pH 6.4, 7.0 and 8.0.

An evaluation of the results showed no differences between the triplicates for each sample evaluated, indicating good repeatability of the process and analysis methodology. The native enzyme activity was affected by the pH of homogenization and by the temperature in which the activity was measured. Therefore, the effect of HPH on β-galactosidase activity was evaluated for each temperature and pH, comparing the results obtained for the native and homogenized samples.

Homogenization of β-galactosidase (up to 150 MPa) at pH 7.0 did not change the enzyme activity measured at 30ºC. When the activity was measured at 5 and 45ºC and the β-galactosidase homogenized at pH 6.4 or 8.0, the enzyme presented a slight activity loss after homogenization at pressures of up to 100 MPa, and a significant, intense loss of activity after treatment at 150 MPa, reducing the β-galactosidase activity by up to 70%. Therefore the stability of β-galactosidase to homogenization is dependent on pH, which can be attributed to the fact that the effects of HPH are dependent on the enzyme conformation, which changes as a function of the positive and negative charge equilibrium of the molecule.

Previous results indicated that HPH may improve [17,18,19, 31,34], have no effect [33], or reduce [15,34] the activity of enzymes, depending on the type of enzyme and the level of pressure applied [17,18].

The results obtained for β-galactosidase at neutral pH (close to the pH of milk) highlight that it is possible to produce high pressure homogenized milk with low lactose content by adding the enzyme to the refrigerated raw milk prior to homogenization.

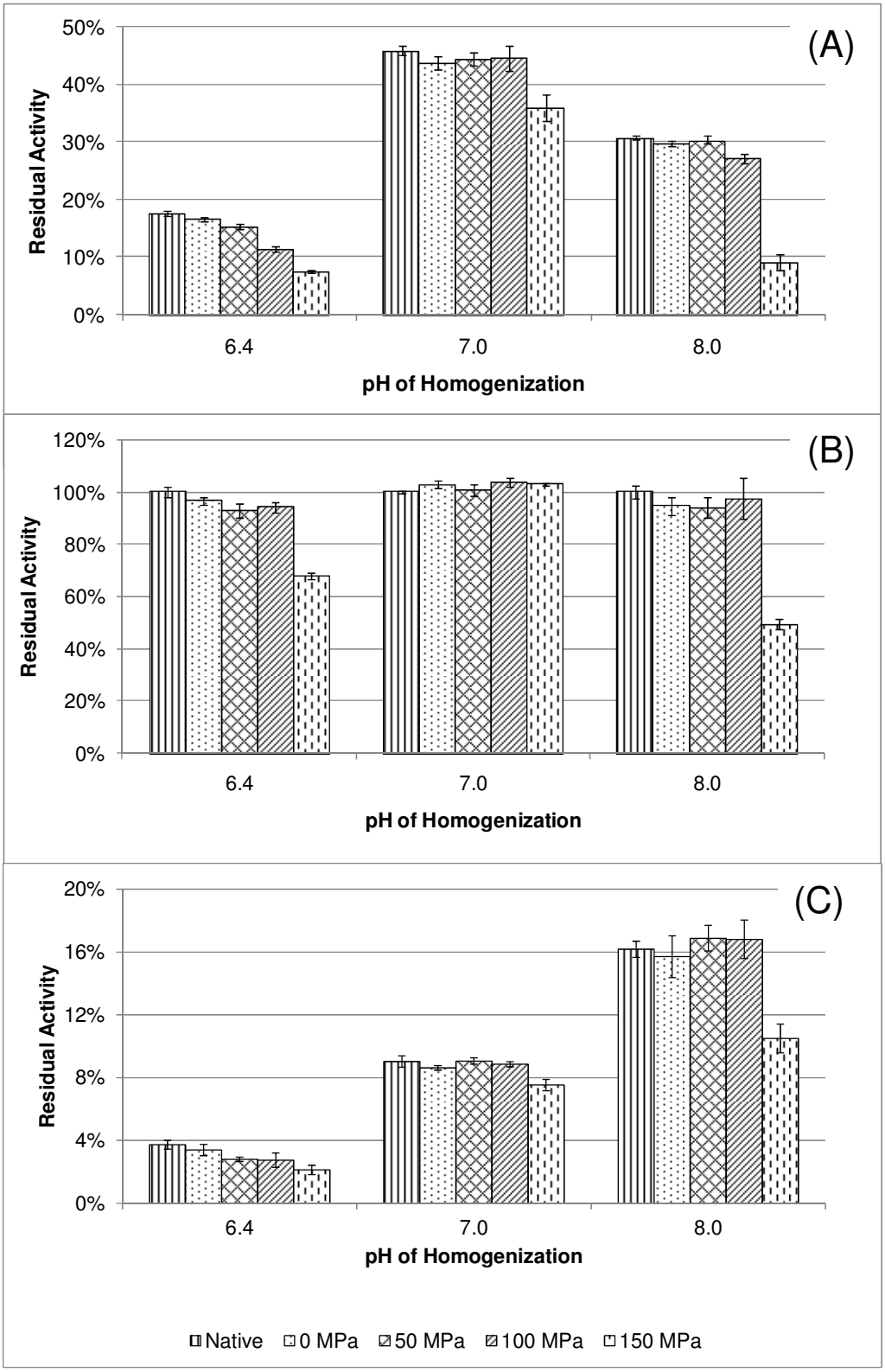

Figure 6.3. Effects of HPH between 0 and 150 MPa on the β-galactosidase activity measured at 5ºC(A), 30ºC(B) and 45ºC(C)

The results obtained in the present work (i.e., a buffer model system) can be extended to milk system, because the main factor that stabilizes β-galactosidase activity in milk is the milk's pH and its buffering capacity [21]. The presence of Mn^{2+} in milk could also contribute to an improved enzyme activity [20]; however, Mn^{2+} was not added to the buffer solution to simulate milk system in the present study. Also, it is important to consider the possible protective effect of the milk constituents (e.g., proteins and fat) on the maintenance of β-galactosidase configuration. Although some research had studied the protective effect of milk on microorganisms during the homogenization process [3], no data describing milk's protective effect on enzymes was published. However, as HPH did not affect the β-galactosidase activity in phosphate buffer as demonstrated in this study, it is unlikely that with the additional potential protective effects of milk, the enzyme activity would be further reduced after the homogenization process.

The results of β-galactosidase activity after HPH can be especially interesting when the milk is to be used to produce fermented dairy products with low lactose content, exploiting the advantages of HPH [2, 17, 25, 30] in the preparation of milk for fermentation in response to the demand for low-lactose dairy products as a result of lactose intolerance disease. It should be emphasized that fermentation alone does not guarantee lactose free or low lactose content dairy [6].

A correlation between the UV absorption peak and β-galactosidase activity was observed, bacause no changes in UV absorption were observed at pH 7.0 (for samples homogenized up to 150 MPa), and higher UV absorption was observed for samples homogenized at 150 MPa when the buffer solution was pH 6.4 (*Figure* 6.2). These results may indicate that the active site of the β-galactosidase was highly affected by the changes in hydrophobic groups (tryptophan and tyrosine residues at pH 6.4). On the other hand, at pH 8.0, although higher UV-absorption was observed at pressures above 50 MPa, the enzyme activity only reduced after homogenization at 150 MPa. This may be related to changes in the spatial configuration of the enzyme caused by the alkaline pH, changing the effects of HPH on the β-galactosidase configuration and active sites.

β-galactosidase activity was measured after one day at 8ºC with the aim of evaluating the enzyme stability under this condition. The native and homogenized enzyme at 50 and 150 MPa (pressures that promoted minimum and maximum changes in the β-galactosidase activity) were evaluated. The results are shown in *Table 6.2*.

The native β-galactosidase activity was affected by refrigerated storage at almost all the pH values evaluated, with significant reduction in activity. The enzyme only remained active when stored at pH 7.0 and the activity measured at the optimum temperature, indicating that β-galactosidase has high stability and activity at pH 7.0. On the contrary, the native enzyme has low stability during storage in buffer at pH values of 6.4 and 8.0.

The pH of the solution, the pressure applied and the temperature in which the activity was measured affected the activity of β-galactosidase after one day of storage. However, for any of the conditions tested, after one day of storage the activity of the homogenized enzyme was higher than the activity of the native one stored under the same conditions, showing that homogenization did not improve the enzyme storage stability.

After one day at pH 8.0, the 150 MPa homogenized enzyme and the native enzyme had different activity for the three temperatures evaluated. This indicates that, at this pH, the loss in activity caused by HPH at 150 MPa was permanent. On the other hand, the activity at 5ºC (enzyme at pH 6.4 and 7.0), 30ºC (enzyme at pH 7.0) and 45ºC (enzyme at pH 6.4) was similar for the homogenized (50 and 150 MPa) and native enzymes after one day of storage. Therefore the effects of homogenization may be reversible under these conditions.

No correlation could be made with the results for residual activity and UV-absorption after one day of storage. As previously described, just diluting the enzyme and storing were sufficient to considerably change the UV-absorption of the native enzymes, which may have overlapped with the different effects of homogenization.

Table 6.2. Residual β-galactosidase activity at 5, 30 and 45ºC after one day of storage (8ºC) at pH 6.4, 7.0 and 8.0

		pH 6.4		**pH 7.0**		**pH 8.0**	
T (ºC)	**Sample**	**0 day**	**1 day**	**0 day**	**1 day**	**0 day**	**1 day**
	Native	17.7 ± 0.5%^{a*}	10.5 ± 0.1%^{d}	45.9 ± 0.8%^{a}	26.7 ± 0.5%^{c}	30.7 ± 0.3% ^{a}	26.5 ± 0.1%^{c}
5ºC	50 MPa	15.3 ± 0.4%^{b}	8.6 ± 0.6%^{d}	44.3 ± 1.1% ^{a}	24.7 ± 0.7% ^{c}	30.2 ± 0.9% ^{a}	25.2 ± 1.3%^{c}
	150 MPa	7.5 ± 0.1%^{c}	7.6 ± 0.5%^{c,d}	35.9 ± 2.4% ^{b}	23.8 ± 2.4% ^{c}	9.1 ± 1.3% ^{b}	14.2 ± 0.9%^{d}
	Native	100 ± 1.8% ^{a}	65.2 ± 0.3%^{b}	100 ± 0.5%^{a}	104.3 ± 1.2%^{a}	100 ± 2.7% ^{a}	92.7 ± 2.1%^{c}
30ºC	50 MPa	92.8 ± 2.6% ^{a}	51.6 ± 0.6%^{c}	100.7 ± 2.1% ^{a}	96.4 ± 2.8% ^{a}	93.9 ± 3.9% ^{a,c}	90.6 ± 0.5%^{c}
	150 MPa	67.9 ± 2.6% ^{b}	47.1 ± 2.3%^{c}	103.1 ± 1.8% ^{a}	95.1± 4.3% ^{a}	49.3% ± 1.8% ^{b}	53.5 ± 1.2%^{d}
	Native	3.7 ± 0.3% ^{a}	1.9 ± 0.1%^{b}	9.0 ± 0.4% ^{a}	7.2 ± 0.4%^{b}	16.2 ± 0.5% ^{a}	8.8 ± 0.6%^{b,c}
45ºC	50 MPa	2.8 ± 0.1% ^{a}	1.7 ± 0.1%^{b}	9.1 ± 0.2% ^{a}	6.0 ± 0.1%^{c}	16.9 ± 0.8% ^{a}	7.3 ± 0.5%^{c}
	150 MPa	2.1 ± 0.3% ^{b}	1.8 ± 0.1%^{b}	7.5 ± 0.4% ^{b}	5.8 ± 0.2%^{c}	10.5 ± 0.9% ^{b}	5.7 ± 0.3%^{d}

* Different letters mean significant differences in the results ($p < 0.05$); the data was evaluated individually for each pH and temperature of activity

6.3.3. High pressure homogenization of β-galactosidase with an inlet temperature of 20 ºC

The effect of an inlet temperature of the homogenizer at room temperature (20ºC) on the activity of the β-galactosidase (pH 7.0) was evaluated. An inlet temperature of 20ºC was chosen considering the low thermal stability of the β-galactosidase studied and the expected heating promoted by the homogenization process. The evaluation of the temperature during processing showed that the maximum temperature reached was 40.1º C at 150 MPa.

The results obtained for enzyme activity after HPH are shown in *Figure 6.4*. The results obtained at 5ºC and 45ºC showed a significant reduction in β-galactosidase activity for each increment of pressure. At 30ºC, no significant differences were observed between the native and homogenized enzymes up to 100 MPa, but a reduction of around 80% was observed after HPH at 150 MPa.

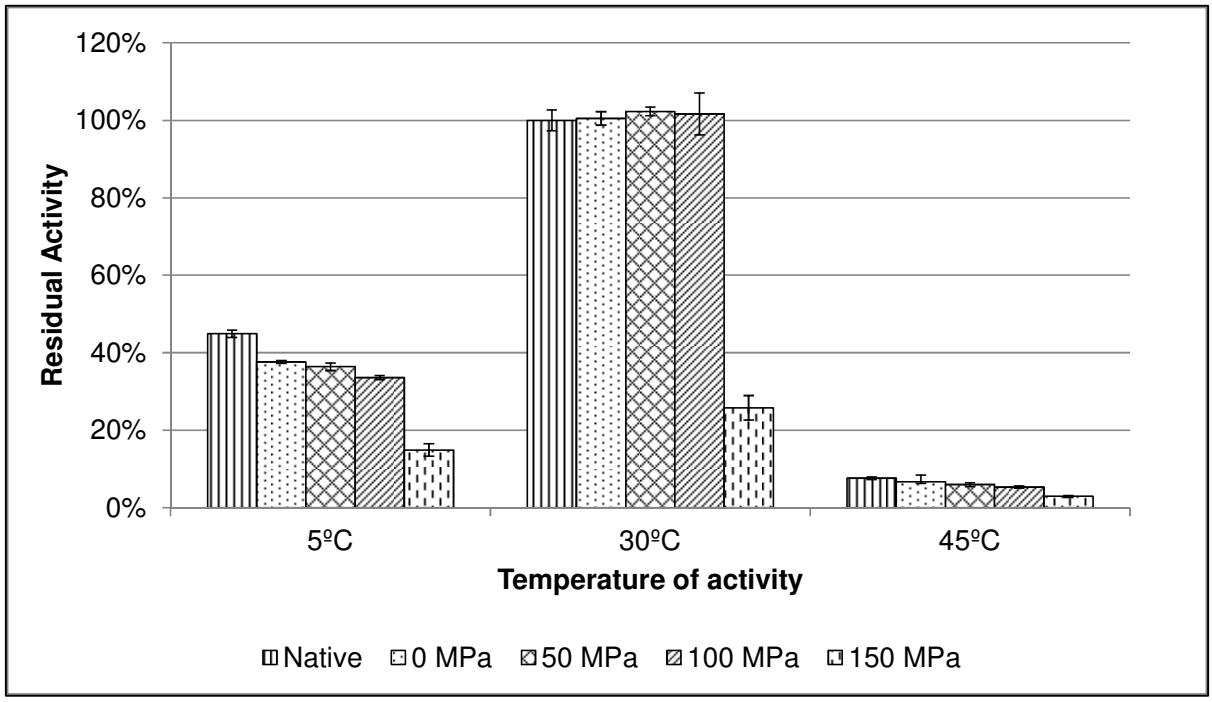

Figure 6.4. Residual activity of β-galactosidase homogenized at pH 7.0 and at room temperature (20ºC)

Comparing these results with those obtained for β-galactosidase homogenized at a refrigerated temperature (*Figure 6.3*), it can be seen that the activity of the enzymes homogenized at room temperature was lower than that of those homogenized at refrigerated temperatures, for all the pressures evaluated (activity at 5ºC and 45ºC) and for the samples homogenized at 150 MPa (activity at 30ºC). These results indicated that the process at 20ºC negatively affected the activity of β-galactosidase, which could be associated with the sum of the effects of HPH with those of the heating caused by shear, especially during homogenization at 150 MPa, since the enzyme had low thermal stability. Therefore it was concluded that homogenization at room temperature was deleterious for the enzyme, and that no advantages were found in homogenizing the enzyme under this condition.

The residual activity was measured after one day of refrigerated storage to evaluate if the activity loss caused by HPH at 20ºC was reversible and the results are shown in *Table 6.3*.

Table 6.3. Residual β-galactosidase activity after homogenization at an inlet temperature of room temperature and one day of storage

Temperature of		**time of storage at 8ºC**	
activity (ºC)	**Sample**	**0 day**	**1 day**
5.0	Native	44.9 ± 1.0%[a]	27.1 ± 1.8%[d]
	50 MPa	36.4 ± 1.0% [b]	22.1 ± 0.4%[e]
	150 MPa	14.9 ± 1.7% [c]	14.8 ± 0.7%[c]
30.0	Native	100.0 ± 2.7% [a]	89.9 ± 1.9%[c]
	50 MPa	102.3 ± 1.1% [a]	86.1 ± 0.6%[c]
	150 MPa	25.8 ± 3.2% [b]	53.2 ± 1.0%[d]
45.0	Native	7.6 ± 0.3% [a]	6.8 ± 0.5%[b,c]
	50 MPa	6.0 ± 0.5% [b]	5.4 ± 0.3%[b]
	150 MPa	2.9 ± 0.3% [c]	3.4 ± 0.5%[c]

***** Different letters mean significant differences in the results ($p < 0.05$); the data was evaluated individually for each temperature of activity

The results indicated that the native and homogenized β-galactosidases at 50 MPa showed an additional loss after one day of storage. On the contrary, samples homogenized at 150 MPa showed no change in the activities measured at 5 and 45ºC after the storage period and, when the activity was measured at 30ºC, an increase in residual activity was observed, which may indicate that the inactivation caused by HPH at room temperature was partially reversible. On the other hand, a comparison of the results obtained for the residual activity of the native and homogenized β-galactosidases at pH 8.5 and 20ºC after one day of storage, only indicated no differences between the activities when it was measured at 5ºC, whereas under all the other conditions evaluated, the enzymes homogenized at 20ºC presented lower enzyme activity.

6.3. Conclusion

The stability of the β-galactosidase during HPH was dependent on the pH and homogenization pressure, being highly stable at pH 7.0, with no changes in the enzyme activity at 30ºC after homogenization at pressures up to 150 MPa. Considering that milk buffering ability is the main factor that affects β-galactosidase activity, the results observed in this present work may indicate that HPH can be used to process milk with added β-galactosidase, with the aim of producing milk or dairy products with low lactose content.

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Capítulo 7. Changes in Commercial Glucose Oxidase Activity by High Pressure Homogenization

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Resumo

A homogeneização à alta pressão (HAP) tem sido descrita como um processo capaz de alterar a atividade e estabilidade de enzimas. Este estudo investigou o efeito do processo na atividade de uma glicose oxidase (GO) comercial. As soluções de enzima foram preparadas em pH 5,0, 5,7 e 6,5 e processadas às pressões de 50, 100 e 150 MPa. O efeito da HAP foi determinado pelas modificações na atividade residual da enzima medida a 15, 50 e 75ºC imediatamente após a homogeneização e após um dia de estocagem. Os resultados mostraram que a homogeneização à baixa pressão (50 MPa) reduziu a atividade relativa da GO em todas as temperaturas testadas quando as amostras foram homogeneizadas em pH 5.0, entretanto, uma recuperação relativa na atividade enzimática foi observada após homogeneização em pressões ≥ 100 MPa. Para amostras processadas em pH 5,7 a homogeneização a 100 MPa reduziu a atividade relativa da enzima a 15 e 50ºC; por outro lado, foi observado um aumento de 25% quando a atividade foi medida a 75ºC após a HAP a 150 MPa. Para amostras homogeneizadas em pH 6,5 o processo reduziu a atividade da GO a 15ºC e praticamente não alterou a atividade enzimática quando esta foi medida a 50 e 75ºC. Após um dia, a atividade relativa da GO homogeneizada aumentou em até 400%, quando comparada com a enzima nativa estocada nas mesmas condições. Estes resultados confirmaram que a HAP alterou a atividade da GO, sendo capaz de promover ativação ou inativação enzimática. Esta mudança de atividade está possivelmente associada às modificações contínuas na estrutura da enzima causada pelas diferentes pressões de homogeneização e pH das soluções. Adicionalmente, o ganho de estabilidade da GO em solução torna a HAP uma ferramenta interessante para melhoria do desempenho da GO, permitindo a expansão das aplicações desta enzima na indústria de alimentos.

Keywords: Glicose oxidase; ultra alta pressão de homogeneização; processo não térmico, atividade enzimática.

Abstract

High pressure homogenization (HPH) has been described as a process able to changes enzyme activity and stability of enzymes. This study investigated the HPH effects on commercial glucose oxidase (GO) activity. Enzyme solutions at pH 5.0, 5.7 and 6.5 were processed at pressures 50, 100, and 150 MPa. The HPH effects were determined by the enzyme residual activity measured at 15, 50 and 75ºC immediately after homogenization and after one day of storage. Results showed that low pressures (50 MPa) reduced the GO relative activity at all temperatures evaluated when samples were homogenized at pH 5.0. However, a relative recovery of enzyme activity was observed when homogenization was carried out at pressures of ≥ 100 MPa. For samples processed at pH 5.7, the homogenization at 100 MPa reduced the relative enzyme activity at 15 and 50ºC. On the contrary, a 25% improvement on GO relative activity at 75ºC was observed after homogenization at 150 MPa. For samples homogenized at pH 6.5, the process continuously reduced the GO relative activity at 15ºC and almost no changes were observed when activity was evaluated at 50 and 75ºC. After one day, the GO relative activity of homogenized samples could increase up to 400%, as compared to the native one stored under the same condition. The results confirmed that HPH changes the GO activity, being able to increase or decrease it. This activity change may be associated to continuous modifications in enzyme structure due to homogenization pressure and pH of solution. Additionally, the GO relative stability increase in aqueous solution highlights HPH as an interesting tool to improve GO performance, expanding the potential application range of glucose oxidase in food industry.

Keywords: Glucose oxidase; Ultra-high pressure homogenization; Non-thermal process, Enzyme activity

7.1. Introduction

Glucose oxidase (GO) (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is a dimeric glycoprotein, consisting of two polypeptide chains covalently linked by disulfide bounds (Bankar, Bule, Singhal, & Ananthanarayan, 2009), with manose, hexosamine and glucose in its structure and a FAD as cofactor. The enzyme catalyzes the oxidation of β-D-glucose to gluconic acid by using molecular oxygen as an electron acceptor with simultaneous hydrogen peroxide production (Fiedurek, & Gromada 1997; Bankar et al., 2009). The enzyme is produced by yeasts and molds, *Aspergillus niger* being the main microorganism used for GO production (Pluschkell, Hellmuth, & Rinas, 1996).

The GO has been adopted to remove oxygen from food, improving color, flavor and shelf life. It can also be applied in eggs glucose removal before pasteurization or drying, avoiding browning by Maillard reaction (Bankar et al., 2009). Furthermore, its use in the biosensor area is growing, especially for the quantitative determination of D-glucose in samples such as body fluids, foodstuffs, beverages, and on fermentation processes (Rauf et al., 2006; Bankar et al., 2009).

The GO is an unstable enzyme, being denatured by temperature, extremes of pH and also in aqueous solution, with a half-life of 30 minutes (Bankar et al., 2009). Therefore, GO stabilization against these destructive factors is required to improve its commercial applications. Immobilization (Rauf et al., 2006; Altikatoglu, Basaran, Arioz, Ogan, & Kuzu, 2010), genetic engineering (Zhu et al., 2010) and conjugation (Altikatoglu et al., 2010) are techniques applied to improve GO stability.

High pressure homogenization (HPH) technology – also known as dynamic high pressure (Lacroix, Fliss, & Makhlouf, 2005; Liu et al., 2010) and ultra-high pressure homogenization (Tribst, Franchi, & Cristianini, 2008) – is an emerging technology developed for food preservation with minimum nutritional and sensory damages (Tribst Franchi, De Massaguer, & Cristianini 2011; Franchi, Tribst, & Cristianini, 2011).

This process was previously studied to inactivate microorganisms, mainly in model systems, milk and juices. These studies were carried out using vegetative

bacteria (Tahiri, Makhlouf, Paquin, Fliss, 2006; Campos, & Cristianini, 2007; Briñez, Roig-Sagues, Herrero, López, 2007; Tribst, Franchi, & Cristianini, 2008; Franchi, Tribst, & Cristianini, 2011;Pedras, Pinho, Tribst, Franchi, & Cristianini, 2012; Velázquez-Estrada, Hernández-Herrero, Guamis-López, Roig-Sagués, 2012), yeasts (Tahiri et al., 2006; Suárez-Jacobo, Gervilla, Guamis, Roig-Sagués, & Saldo, 2010; Franchi, Tribst, & Cristianini, 2011, Velázquez-Estrada et al., 2012) and molds (Tahiri et al., 2006; Tribst et al., 2009; Suárez-Jacobo et al., 2010, Tribst et al., 2011). The initial published works highlighted that HPH was not capable of causing sublethal inactivation on microorganisms (Wuytach, Diels, & Michiels, 2002; Diels, Taeye & Michiels, 2005; Briñez et al., 2007); however, more recent data showed that HPH can have a synergistic action with a mild thermal process for inactivation of *A. niger* (Tribst et al., 2009) and of *Bacillus cereus* and *Bacillus subtilis* (Chaves-López, Lanciotti, Serio, Paparella, Guerzoni, & Suzzi, 2009).

Furthermore, HPH cause changes in configuration of proteins, polysaccharides and suspended particles, using pressures up to 200MPa (Augusto et al., 2012a; Augusto et al., 2012b, Lacroix, Fliss, & Makhlouf, 2005). HPH was previously described as a process capable (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009; Liu et al., 2009a,b; Tribst, & Cristianini, 2012b; Tribst, Augusto, & Cristianini, 2012a,b) or otherwise (Tribst, & Cristianini, 2012b) of changing enzyme activity and stability, the effects normally being associated with the individual enzyme being evaluated and with the homogenization pressure applied (Liu et al., 2009a,b). Data about pectin methyl esterase indicated that homogenization was just able to inactivate the enzyme (Lacroix, Fliss, & Makhlouf, 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Béltran, 2009), while results obtained for polyphenol oxidase showed that HPH causes an activity increase between 120 and 160 MPa (Liu et al., 2009a,b). Data obtained for α-amylase (Tribst, & Cristianini, 2012b) and trypsin (Liu et al., 2010) showed no changes on enzyme activity, however, an increase on trypsin thermal stability was observed (Liu et al., 2010). On the contrary, data of neutral protease (Tribst, Augusto, & Cristianini, 2012a) and amyloglucosidase (Tribst, & Cristianini, 2012b) revealed that these enzymes can be activated or inactivated depending on the homogenization pressure

applied, pH of enzyme solution and the temperature of activity measurement (Tribst, Augusto, & Cristianini, 2012a; Tribst, & Cristianini, 2012b). When passing through the homogenizer, the sample is submitted to pressure increase and decrease, and shear stress whose mechanical energy may induce molecular unfolding. When an enzyme is unfolded, both activation and inactivation are expected. This conformational change can expose the active site and increase its activity or prevent its contact with the substrate, reducing enzyme activity. Therefore, these results indicated that is not possible to establish a rule about the homogenization effects on enzymes.

This research studied the effect of high pressure homogenization at different pressure levels and pH on GO activity at optimum (50ºC) and non-optimum temperatures (15 and 75ºC) and also the process effects on GO stability after refrigerated storage.

7.2. Material and methods

7.2.1. Enzyme characteristics

The glucose oxidase used in this experiment was a commercial enzyme from (Prozyn Biosolutions®, São Paulo, Brazil, www.prozyn.com - batch number I – 368592910). The enzyme was obtained from *A. niger* fermentation (molecular weight about 150 KDa, containing 16% of carbohydrates) and had pH stability between 3.5 and 7.0 and optimum activity at 50^{o}C.

7.2.2. Enzyme activity

The GO activity was determined by using the method described by Kona, Quereshi, & Pai (2001) with a few modifications: 400µL of enzymatic solution (0.3 g of dried enzyme per liter of 0.1 M acetate buffer, pH 5.0 with 0.02 g.L^{-1} of sodium nitrate) was added to 400 µL of glucose solution (4 g.L^{-1}) and to 1.2 mL of 0.1 M acetate buffer pH 5.0. The reaction was carried out at 50ºC for 30 minutes. Then, 1.5 mL of DNSA solution was added followed by heating at 100ºC for 5 minutes. A control

sample was obtained using a similar procedure, but with no addition of GO solution. After heating, the samples were cooled and 6.5 mL of 0.1 M acetate buffer (pH 5.0) was added. Their absorbance values were measured at 547 nm in a spectrophotometer DU 800 (Beckman Coulter®, Brea, CA).

The standard curve was obtained by using glucose solution at concentrations of 0.5, 1, 2, 3, 4 and 6g.L^{-1} prepared using 0.1 M acetate buffer pH 5.0 in triplicate. The glucose reacted with DNSA following the procedure described above and absorbance was measured at 547 nm in triplicate.

The absorbance of the samples was converted to glucose concentration by standard curve. GO activity was calculated by the difference of glucose concentration in the control and in GO samples. One enzyme unit was defined as the amount of enzyme which converts 1 µg of glucose per minute. The final GO activity was calculated per gram of commercial dried enzyme.

7.2.3. Optimum pH and temperature

The activity of native GO was evaluated at pH 3.6, 4.3, 5.0, 5.7, and 6.5, using 0.1M acetate buffer (pH 3.6 – 5.7) and 0.1 M citrate-phosphate buffer (pH 6.5). The effect of temperature was evaluated at 15, 50 and 75ºC. The enzyme activity was measured by the DNSA method, changing the buffer pH and reaction temperature. Standard curves were obtained using buffers of 3.6, 4.3, 5.0, 5.7, to prepare glucose solution at the same concentration described in section 7.2.2. The standard curve characteristics are indicated in the *Table 7.1*.

Table 7.1. Glucose standard curve at different pH

pH	Glucose Standard Curve	R^2
3.6	$[\mu g_{glucose}] = 22105.00*abs_{547nm} + 878$	>0,99
4.3	$[\mu g_{glucose}] = 20859.10*abs_{547nm} + 6.16$	>0,99
5.0	$[\mu g_{glucose}] = 3473.20*abs_{547nm} + 96.05$	>0,99
5.7	$[\mu g_{glucose}] = 3092.20*abs_{547nm} + 98.17$	>0,99
6.5	$[\mu g_{glucose}] = 9744.10*abs_{547nm} + 4.67$	>0,99

The optimum pH and temperature were chosen considering the highest activity measured in the experiment. At this condition (optimum pH and temperature), 100% of residual activity was established. For the other samples evaluated, the residual activities were calculated using *Equation 7.1*.

Residual activity (%) = (Activity at non ideal condition/ optimum activity) · 100 (*Equation 7.1*)

7.2.4. High pressure homogenization and activity of homogenized GO

A Panda Plus high pressure homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the tests. The equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200MPa. The equipment operates at a flow rate of $9L \cdot h^{-1}$.

A volume of 2L of the GO solution (0.3 g of dried enzyme per liter of 0.1M buffer, with 0.02 $g.L^{-1}$ of sodium nitrate - activity 18.9 $U.mL^{-1}$) at 23.0ºC (pH 5.0, 5.7 and 6.5) was homogenized under pressures of 50, 100 and 150 MPa. A control of process (obtained by pumping the enzyme solution through the homogenizer with no pressure applied) was evaluated, since previous results indicated that the sample pumping on the homogenizer was able to change the activity of a neutral protease (Tribst, Augusto, & Cristianini, 2012). Samples (50 mL) were collected, and unprocessed GO (native) was evaluated, as previously described by Tribst, & Cristianini (2012a). The sample temperatures were measured using a type T thermocouple inserted in buffer solution before and immediately after the homogenization process. The residence time was determined as the time spent between the enzyme inlet in the homogenizer and the end of sample collection.

The enzyme activities were performed at pH 5.0 and temperatures of 15ºC, 50ºC and 75ºC. The assays were carried out immediately after HPH and after 24 h of refrigerated storage at 8ºC for native and GO homogenized at 50 and 150 MPa. The 8ºC was chosen because it is a common temperature used for food preservation in Brazil.

7.2.5. Activity of high pressure homogenized GO at high inlet temperature

A sample of GO solution (pH 5.0, 5.7 and 6.5) was homogenized (0, 50, 100 and 150 MPa) at the inlet temperature of 50ºC, using the same procedure described for enzyme at room temperature. The temperature of 50ºC (optimum temperature for enzyme activity) was chosen to evaluate if the effects of HPH on enzyme improve at this condition. The GO activity was performed at pH 5.0 and 50ºC immediately after homogenization and after 24 h under refrigerated storage at 8ºC (native and samples homogenized at 50 and 150 MPa).

7.2.6. Statistical analysis

The analysis of variance (ANOVA) was carried out to compare the effects of the different treatments, and the Tukey test was used to determine the differences between them at a 95% confidence level. The statistical analyses were carried out using the STATISTICA 5.0 software–(StatiSoft, Inc., Tulsa, Okla., U.S.A.). All the processes and the determination of glucose oxidase activity were carried out in triplicate. The experiments were carried out on different days using different suspensions of enzymes. The results were represented as the mean ± standard deviation.

7.3. Results and discussion

7.3.1. Optimum pH and temperature

Figure 7.1 shows the native GO activity at different pH and temperature. The pH 5.0 and a temperature of 50ºC were established as the optimum GO conditions, i.e., the condition of GO highest activity. Under this condition the activity was 1,888,444 $U.g^{-1}$, which was defined as 100% of residual activity.

An intense activity reduction (almost 80%) was observed at non optimum pH, asserting that GO activity is highly affected by this parameter. On the contrary, the

reaction temperature significantly reduced the enzyme activity just at 75ºC. This was expected, since GO was previously described as an enzyme with low activity at non-optimum conditions (Bankar et al., 2009).

Considering the results of the *Figure 7.1*, the measurement of GO activity was carried out at pH 5.0 after HPH (optimum pH). Previous results showed that HPH improved enzyme activity at non-optimum temperatures (Tribst, Augusto, & Cristianini, 2012; Tribst, & Cristianini, 2012b) and also enzyme stability at high temperature (Liu et al., 2010). Therefore, the HPH effects on GO activity at different temperatures (15, 50 and 75ºC) were evaluated. Considering that GO are employed in pasteurized food, it would be interesting to keep the enzyme active at high temperatures.

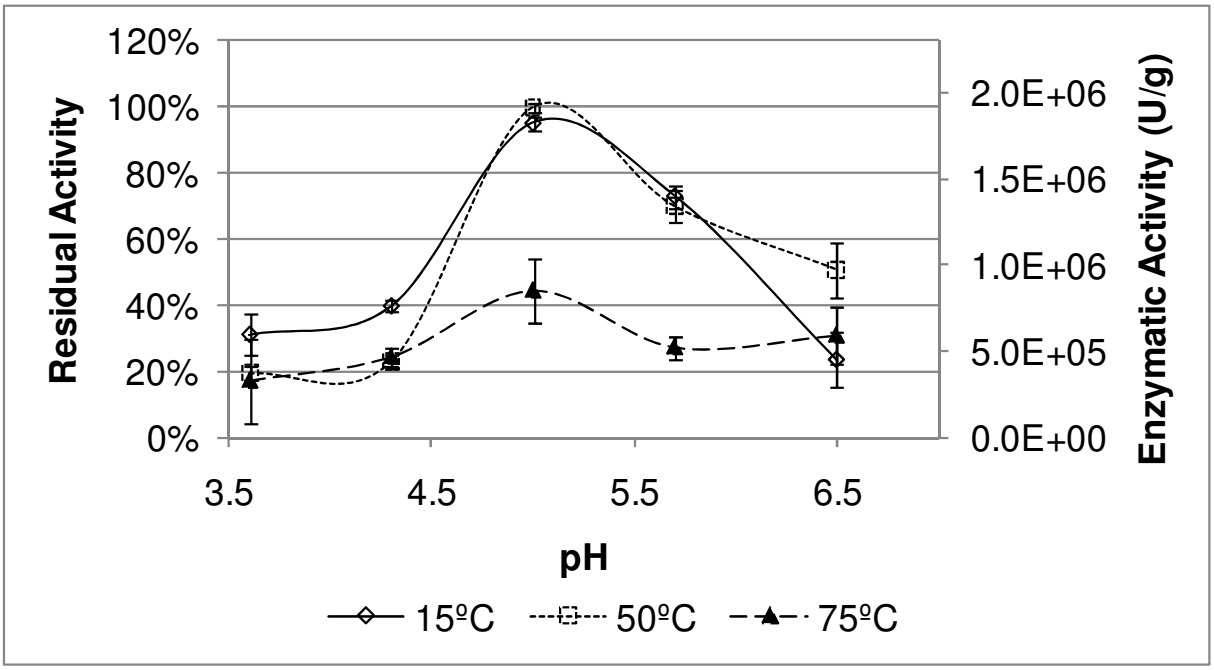

Figure 7.1. Effects of pH and temperature on GO activity

7.3.2. HPH of glucose oxidase at room temperature

The HPH processes were carried out at pressures up to 150 MPa. The fast decompression during the process promotes intense shear and friction with consequent heating of the homogenized fluid. Considering that enzymes can be affected by heating, the sample temperature reached at each pressure was

measured. The residence time at those temperatures was < 10 s. The temperature increment had a linear correlation (R^2 = 0.999) with homogenization pressure applied during the process, being described by *Equation* 7.2.

$$\text{Temperature (ºC)} = 0.14 * P_H + 2.61 \qquad (\textit{Equation } 7.2)$$

With:

P_H = pressure of homogenization at MPa

The pressure increase around 1.4ºC at each 10 MPa and maximum temperature raised was 48.4ºC at 150 MPa. This temperature cannot cause enzyme thermal denaturation. Consequently, all effects observed were attributed to the HPH process. The *Figure 7.2* shows the HPH effects on GO activity measured at different temperatures.

No statistical differences were found between the triplicates of each sample, indicating good repeatability of process and analysis methodology. The pH of homogenization and the temperature of activity measurement impacted on the native enzyme activity. Therefore, the effects of HPH on the GO were evaluated for each temperature and pH, comparing the results obtained for native and homogenized samples.

The activities of homogenized samples at pH 5.0, 5.7 and 6.5 were different for the temperatures evaluated. This indicated that pH altered the enzyme native molecule configuration and, consequently changed the enzyme susceptibility to pressure homogenization. A reduction on GO activity occurred for most conditions studied. On the other hand, 150 MPa at pH 5.7 caused a relative increase of 25% in GO activity (from 50 to 65% of residual activity) measured at 75ºC ($p < 0.05$). This can be advantageous for pasteurized food added by GO, when the enzyme needs to be active during food shelf-life (e.g. GO use in pasteurized juice with pH near to 5.0, e.g. watermelon, banana, to prevent oxidation and activity of PFO during the juices shelf life (Bankar et al., 2009)) or in probiotic yogurt to reduce cell damage caused by oxygen (Da Cruz et al., 2010).

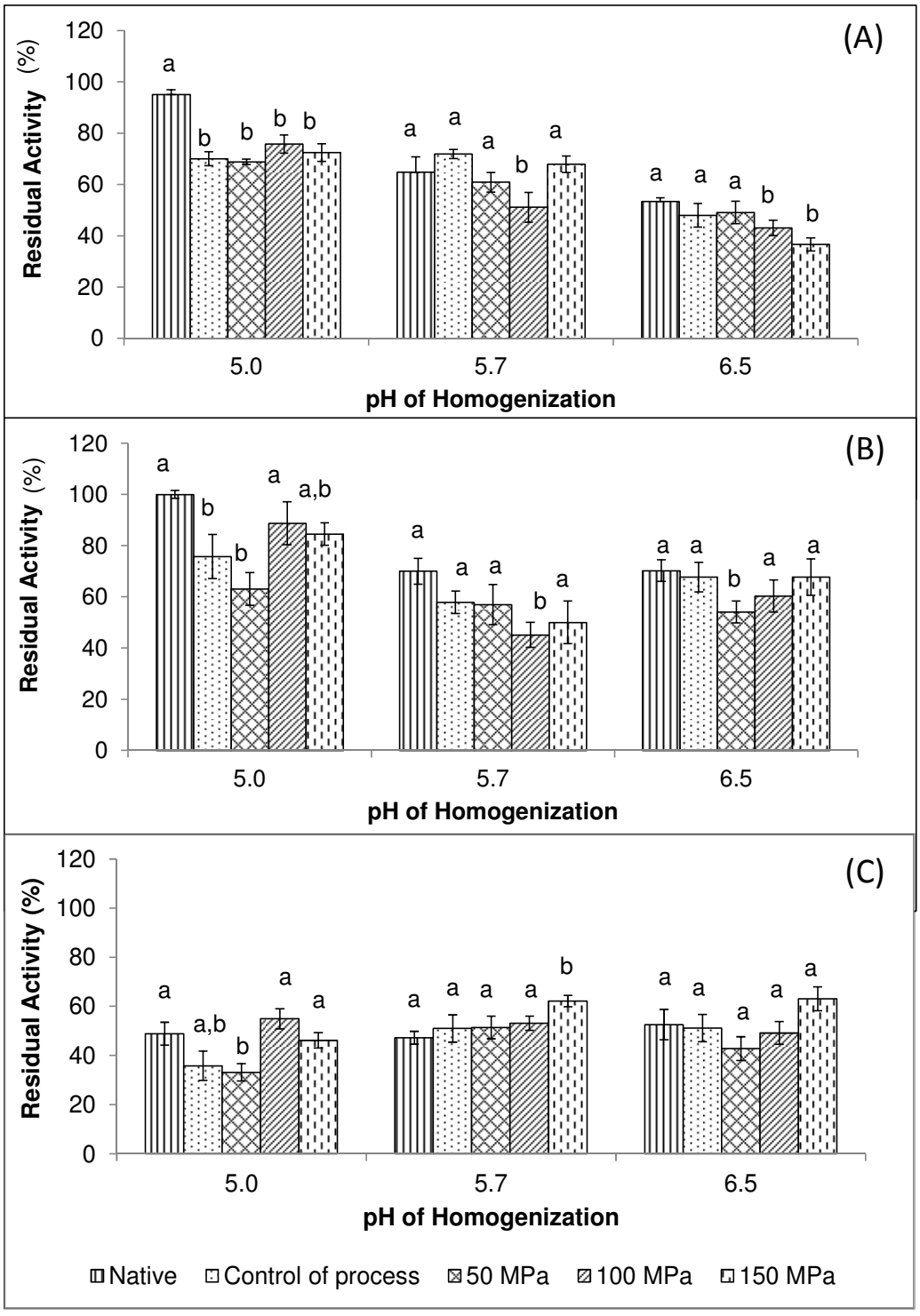

Figure 7.2. Effects of HPH on the GO activity at pH 5.0 measured at 15ºC(A), 50ºC(B) and 75ºC(C). *Different letters mean significant difference ($p<0.05$) between samples evaluated at the same pH and temperature. The results were the means of three GO activity measurements from three HPH assays.*

For samples at pH 5.0, the pumping of GO solution into the equipment with no pressure (control of the process) caused a reduction in its activity, demonstrating that GO is easily denatured. This was also previously observed for neutral protease (Tribst, Augusto, & Cristianini, 2012b) and the effects were attributed to changes in enzyme configuration due to minimum shear during pumping or to the possibility of air incorporation in the fluid with consequent protein denaturation at the air/water interface.

For most conditions, relative low homogenization pressure (50 and 100 MPa) resulted in maximum enzyme activity reduction (up to 35%). Conversely, higher pressure (150 MPa) results on GO activity recovery and samples reached the same activity of the native one (exceptions were samples homogenized at pH 5.0 and 6.5 with activity measured at 15ºC). These results suggest that HPH continuously affected the GO active sites exposure and, consequently, the GO conformation. Also, pH of samples influenced the HPH effects intensity.

7.3.3. Storage effect at 8°C for 24 hours on activity of GO

The GO relative activity was measured after one day of storage at 8ºC aiming to evaluate enzyme stability at this condition. Also, it was used to determine if the changes on GO caused by HPH are transitory or permanent. The *Figure 7.3* exhibits these results.

Native enzyme activity was affected by the pH of storage solution and also by the temperature of activity measurement. Therefore, results were evaluated for each temperature and pH, comparing the results of native and homogenized samples.

The comparison of GO activities (native sample) immediately after preparation (*Figure 7.2*) and after one day at 8ºC (*Figure 7.3*) denoted that storage reduced enzyme activity. This was expected since GO has low stability in aqueous solution (Bankar, 2009).

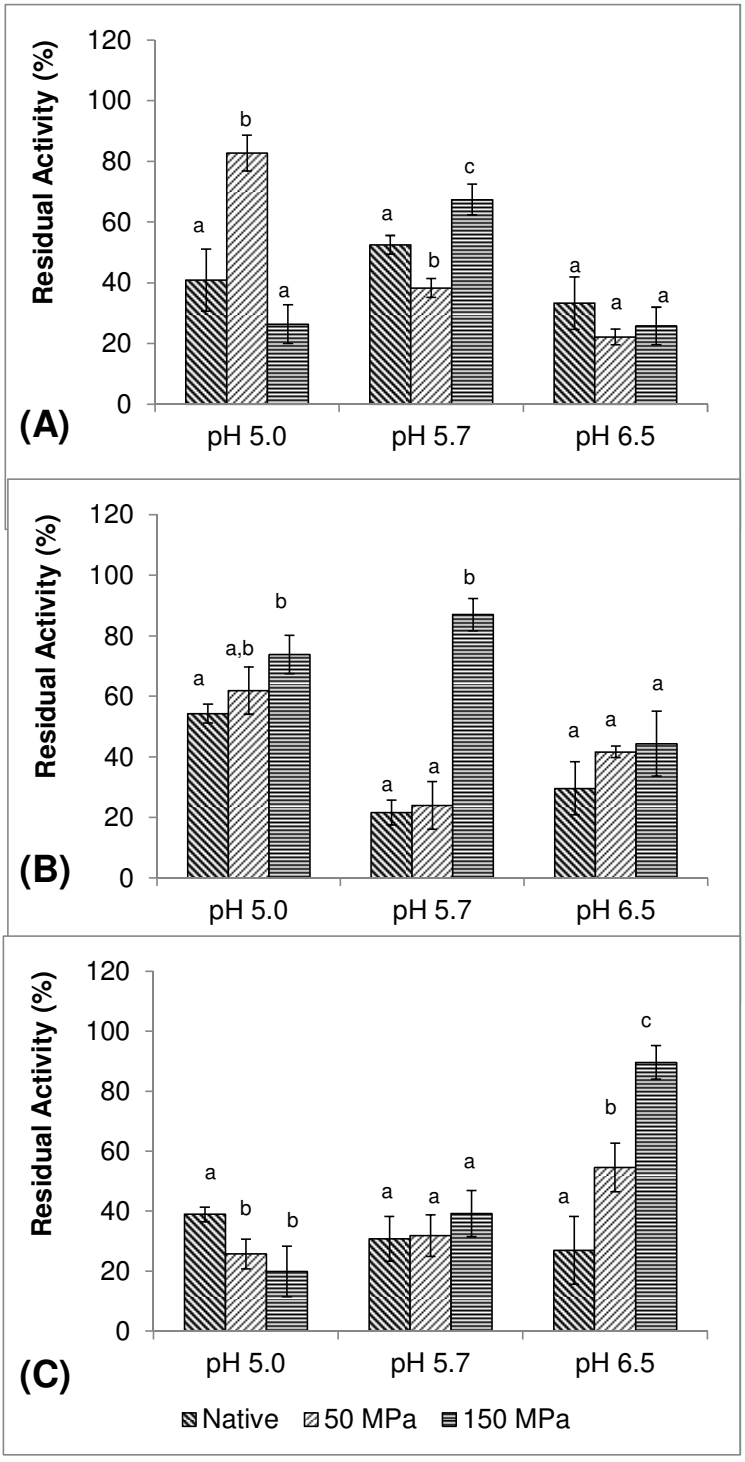

Figure 7.3. Residual activity of homogenized GO after one day of storage at 15ºC(A), 50ºC(B) and 75ºC(C). Different letters mean significant difference ($p<0.05$) between samples evaluated at the same pH and temperature. The results were the means of three GO activity measurements from three HPH assays.

The pH of the solution, pressure applied and the temperature of activity measurement influenced the activity of the enzyme after storage. For samples at pH 5.7 (activity measured at 75ºC) and pH 6.5 (activity at 15 and 50ºC), native and homogenized samples (50 and 150 MPa) had the same activity after one day of storage. However, at other conditions, the activities of native and homogenized enzyme were different. During the storage period, the enzyme could be more or less stable depending on the pH of storage and also the changes caused by HPH. Additionally, the activity of enzyme at different temperatures is dependent on the level of molecular agitation and exposure of active site. Therefore, depending on the changes on enzyme during storage, the enzyme can or cannot change its activity at each evaluated temperature. Considering the results of GO stability during storage, it may be possible to suppose that HPH enzyme did not return to its native configuration after one day of rest. Possibly, after a period, the enzyme configuration reached a stable form different from the native one.

For GO homogenized at 50 MPa, just the samples prepared at pH 5.0 (activity measured at 15ºC) and at pH 6.5 (activity measured at 75ºC) presented activity higher than native. After 150 MPa, on the contrary, some samples had a relative activity increase after storage, with maximum improvement reached at pH 5.7 when activity was measured at 50ºC (4 times increment on residual activity).

These results suggest that HPH can be used to improve enzyme stability during storage in aqueous products. Moreover, the HPH condition can be chosen by the pH of the product and the temperature of the desired activity, i.e. if it is required that GO keep its activity at 15ºC, a previous treatment of 50 MPa at pH 5.0 is able to increase the residual activity of the enzyme by 100%. Therefore, this process can be interesting to increase the shelf-life of products due to oxygen consumption by GO. A potential application is the addition of GO in probiotic yogurts, since GO would consume the oxygen that permeates the package during storage, improving the viability of probiotic cultures (Cruz et al., 2010).

7.3.4. GO homogenization at high inlet temperature

The inlet temperature of 50ºC was chosen since it is the temperature with maximum exposure of active sites of GO. Considering the higher temperature, it was firstly assessed if the temperatures reached during homogenization were able to inactivate the enzyme. The maximum temperature was 57.6ºC at 150 MPa, which is not enough to induce thermal inactivation of GO during the expected residence time (10 s). The lower temperature gain for the sample processed at high temperature compared with sample processed at room temperature can be explained by the tendency of sample temperature equilibrium with the temperature of the equipment (placed at room temperature of 25ºC) and the high relation of equipment/ sample mass.

Figure 7.4 shows the residual activity of GO after homogenization at high temperature.

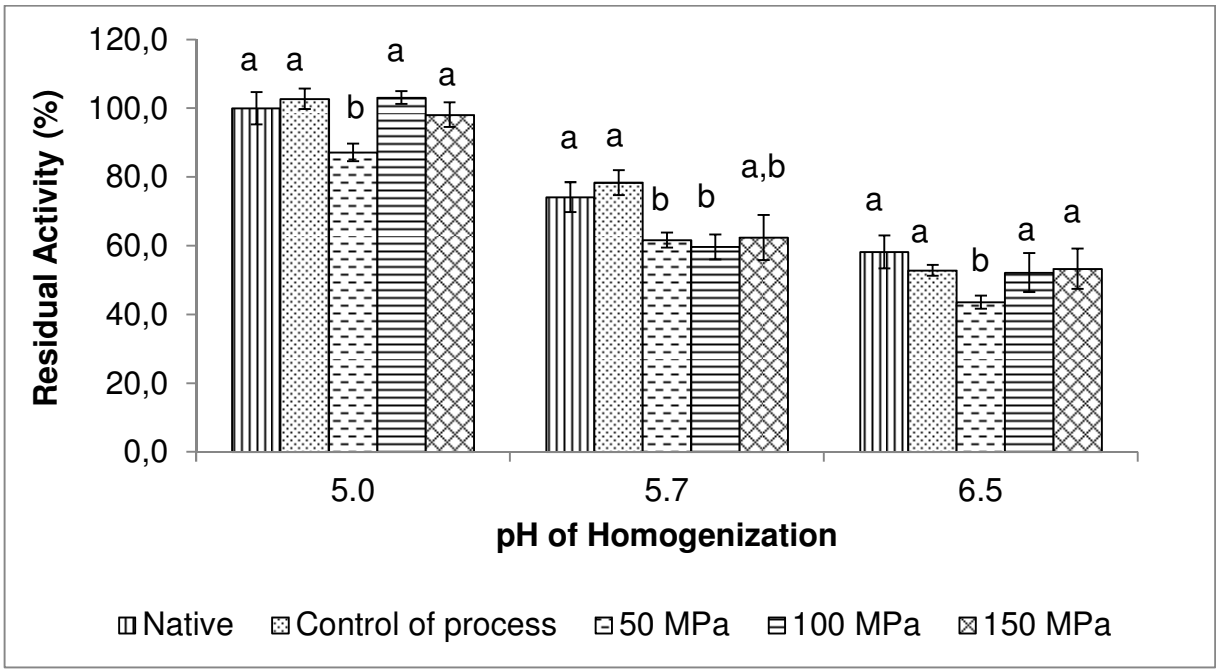

Figure 7.4. Residual activity of homogenized GO at high inlet temperature (50ºC).

Different letters mean significant difference ($p<0.05$) between samples evaluated at the same pH and temperature. The results were the means of three GO activity measurements from three HPH assays.

The pH influenced the activity of native GO, as previously observed in *Figure 7.2.* The results were similar to the homogenized GO at 23.0ºC and 50ºC, with minimum activity after 50 MPa of homogenization at pH 5.0 and 6.5 and minimum activity after 50 MPa and 100 MPa for samples homogenized at pH 5.7 (*Figure 7.4*). However, the evaluation of the residual activity showed that GO became more resistant to HPH at pH 5.0 (optimum pH). On the contrary, the activities hardly changed at pH 5.7 and 6.5, indicating that the inlet temperature did not modify the effects of HPH on GO at these pH.

After one day of storage, the residual activity was measured only for GO homogenized at pH 5.0 (condition that residual activity was different for samples homogenized at different inlet temperatures). The *Figure 7.5* shows these results.

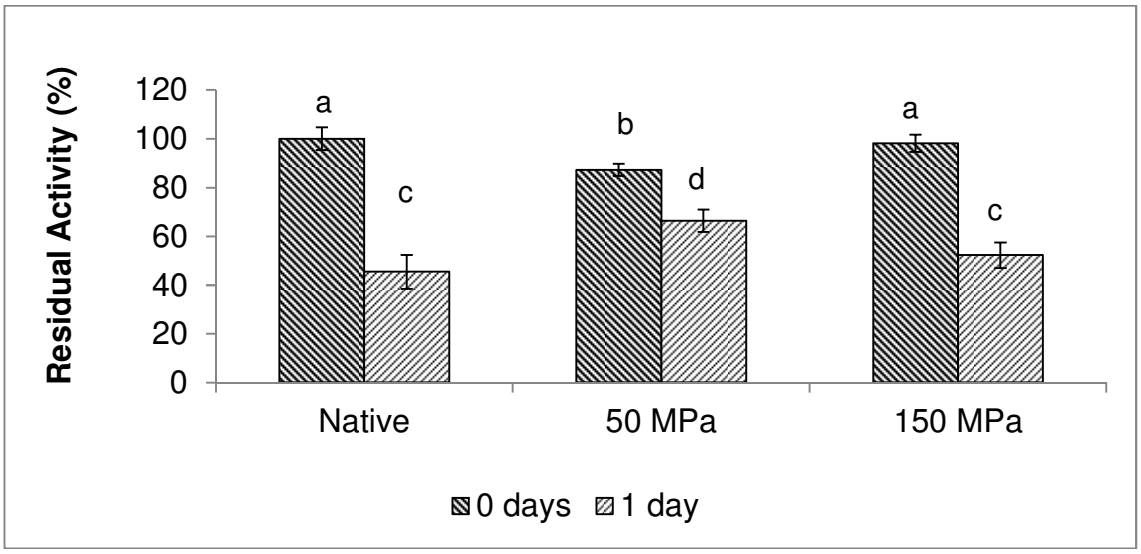

Figure 7.5. Residual activity of homogenized GO at high inlet temperature (50ºC) after one day of storage at pH 5.0 and 8ºC. Different letters mean significant difference (p<0.05) between samples evaluated. The results were the means of three GO activity measurements from three HPH assays.

No significant differences were found between residual activity of native and homogenized GO at 150 MPa (p >0.05). However, the activity of homogenized GO at 50 MPa was slightly higher. On the contrary, the activity was reduced around 10% after one day of storage for sample homogenized at 150 MPa, when compared with

homogenized enzyme at room temperature. This shows that the homogenization carried out at high inlet temperature reduced the storage stability, which may be related to the changes caused by the process due to the sum of homogenization and temperature effects on the enzymes. Consequently, no advantages were observed in homogenizing GO at high inlet temperatures.

7.4. Conclusion

High pressure homogenization is able to alter the glucose oxidase activity and increase its residual relative activity at high temperature after homogenization at pH 5.7 and 150 MPa. Additionally, the HPH can cause an increment up to 400% on GO stability as evaluated after 24 h storage at 8ºC, as compared to the native one stored under the same conditions. Therefore, the HPH may be an interesting tool to increase GO relative stability, improving the potential applications of GO in food industry.

Acknowledgement

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Capítulo 8. Multi-pass high pressure homogenization of commercial enzymes: effect on the activities of glucose oxidase, neutral protease and amyloglucosidase at different temperatures

Trabalho submetido para publicação: TRIBST, A.A.L.; AUGUSTO, P.E.D.; CRISTIANINI, M. Multi-pass high pressure homogenization of commercial enzymes. Trabalho em avaliação.

Resumo

Este trabalho estudou a atividade residual de amiloglicosidase (AMG) a 65 e 80ºC, glicose oxidase (GO) a 50 e 75ºC e protease neutra a 20 e 55ºC após três processamentos sequenciais de homogeneização à alta pressão (HAP) a 200 e 150 MPa (protease neutra e AMG) e a 150 e 100 MPa (GO). Os resultados da amiloglicosidase e da protease neutra mostraram que o aumento máximo na atividade das enzimas foi obtido após a primeira homogeneização a 200 MPa, com um aumento na atividade residual de AMG a 80ºC (de 13 para 21%) e da protease a 20ºC (de 50 para 64%). O efeito dos múltiplos processamentos, entretanto, não resultou em aumento da atividade destas enzimas. Por outro lado, os resultados obtidos para a glicose oxidase mostraram que a HAP a 150 MPa aumentou continuamente a atividade a 75ºC, atingindo uma atividade três vezes maior do que a da enzima nativa após os três processamentos sequenciais no homogeneizador. Além disso, foi observado que dois processos sequenciais a 100 MPa resultaram no mesmo nível de inativação atingido após um processamento único de GO a 150 MPa. Os resultados obtidos neste trabalho indicam que os efeitos das múltiplas HAP são diferentes para cada enzima avaliada e que esta ferramenta pode ser utilizada para melhorar a atividade de glicose-oxidase em altas temperaturas.

Palavras-chave: alta pressão de homogeneização, enzimas comerciais, processos múltiplos, atividade enzimática.

Abstract

This research studied the residual activities of amyloglucosidase (AMG) at 65 and 80ºC, glucose oxidase (GO) at 50 and 75ºC and neutral protease at 20 and 55ºC after 3 passes of high pressure homogenization (HPH) at 200 and 150 MPa (neutral protease and AMG) and at 150 and 100 MPa (GO). The results for AMG and neutral protease showed that the improvement in maximum enzyme activity was reached after one pass at 200 MPa, with an increment in the AMG residual activity measured at 80ºC (activity increased from 13 to 21%) and in the neutral protease residual activity measured at 20ºC (activity increased from 50 to 64%). However, the multiple passes caused no improvement in the activities of the enzymes. To the contrary, the results obtained for GO showed that HPH at 150 MPa continuously improved the activity at 75ºC up to three passes, reaching an activity three times higher than the native sample. Additionally, it was observed that two passes of GO at 100 MPa resulted in the same level of GO activation reached after a single pass at 150 MPa. These results suggest that multiple HPH effects differ for each enzyme evaluated and can be applied to improve GO activity.

Keywords: high pressure homogenization; commercial enzymes; multiple process; enzyme activity

8.1. Introduction

High pressure homogenization (HPH) is an emerging technology developed for food preservation with minimum sensory and nutritional damage (Tribst, Franchi, de Massaguer, & Cristianini 2011; Franchi, Tribst, & Cristianini, 2011). Recently, HPH was also proposed as a physical method to change proteins, being able to alter the activity and / or stability of enzymes (Liu, Liu, Liu, Zhong, Liu, & Wan, 2009a; Liu et al., 2009b; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a,b; Tribst, & Cristianini, 2012a,b) and to change the functional properties of proteins (Subirade, Loupil, Allain, & Paquin, 1998; Bouauina, Desrumaux, Loisel, & Legrand, 2006; Gárcia-Juliá et al., 2008; Keerati-U-Rai, & Corredig, 2009; Luo et al., 2010; Dong et al., 2011; Yuan, Ren, Zhao, Luo & Gu, 2012).

HPH was able to improve (Liu et al., 2009 a,b; Liu et al., 2010; Tribst, Augusto, & Cristianini, 2012a; Tribst & Cristianini, 2012b,c), reduce (Lacroix et al., 2005; Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009; Velázquez-Estrada, Hernández-Herrero, Guamis-López, & Roig-Sagués, 2012; Tribst, Augusto, & Cristianini, 2012b) or not alter (Tribst, & Cristianini, 2012a) the activity and stability of enzymes. The effects of HPH were dependent on the level of pressure homogenization applied, the temperature of the enzyme during the process, the nature of enzyme studied, pH of homogenization and the presence/absence of substrate during homogenization (Liu et al., 2009 a,b; Tribst, & Cristianini, 2012a,b,c; Tribst, Augusto, & Cristianini, 2012, a,b).

In addition, multi-pass homogenization was able to improve the activity of polyphenol oxidase from mushrooms and pears after three HPH passes (Liu et al., 2009a,b), reaching the same level of activity after 2 cycles at 120 MPa as after one cycle at 140 MPa (Liu et al., 2009a). It is important to observe that the lower the homogenization pressure, the smaller the processing costs (equipment and operation). Therefore, the use of multi-passes could be of interest, aiming to optimize processing by high pressure homogenization (maximizing its effect with lower costs).

The changes in enzyme activity/stability were linked with conformational alterations caused to the enzymes by the HPH process, which is able to modify the

quaternary, tertiary and secondary structures (Liu et al., 2009a; Liu et al., 2009b; Liu et al., 2010; Tribst, & Cristianini, 2012b). The main structural effects described are: (i) increase in the hydrophobic sites on the enzyme and exposure of amino acids (Liu et al., 2009b; Liu et al., 2010, Tribst, Augusto, & Cristianini, 2012a), (ii) increase in exposure of SH groups due to unfolding of the protein and a reduction in the total SH content due to new disulphide bonds formation (Liu et al., 2009b; Liu et al., 2010) and (iii) changes in the α-helix, β-sheet and β-turn ratio composition due to alterations of the secondary structure (Liu et al., 2009b). Despite all these changes, the process is apparently unable to alter the enzyme molecular weight (Liu et al., 2010).

In addition to the enzymes, the effects of HPH were also measured on the proteins in general. The results obtained indicated that the process provided enough energy to disrupt the tertiary and quaternary structures of most of the globular proteins (Subirade et al., 1998), induce protein rearrangement and aggregation (Keerati-U-Rai & Corredig, 2009), and increase the protein exposure area (Dong et al., 2011), hydrophobic interactions (Gárcia-Juliá et al., 2008; Luo et al., 2010; Yuan et al., 2012), and reducing power of the hydroxyl radical scavenging (Dong et al., 2011).

Considering previous results, this research studied how multi-pass through the homogenizer changed the activity of three commercial enzymes. It had previously been reported that the selected enzymes could be activated by a single pass through the HPH equipment (Tribst, Augusto, & Cristianini, 2012a; Tribst, & Cristianini, 2012b,c).

8.2. Material and methods

8.2.1. Amyloglucosidase

The amyloglucosidase (AMG) used in these experiments was a commercial enzyme from Prozyn Biosolutions® (São Paulo, Brazil). The enzyme is presented as a yellow powder obtained as a fermentation product from *Aspergillus niger*. It has an

expected molecular weight of 70-90 kDa, and optimum activity in the pH range from 4.4 to 6.0 and temperature range from 40 to 65°C.

The enzymatic activity was determined following the method described by Rami, Das, & Satyanarayana (2000) with a few modifications: 500 µL of enzyme solution (0.1 grams of dried enzyme diluted in one liter of 0.05 M acetate buffer at pH 4.3) was added to 4 mL of a 0.5% (w/v) soluble starch (for analysis degree with purity of 99.6%, (Synth, Brazil) solution. The reaction was carried out at 65 and 80ºC for 10 minutes and stopped by the addition of 3 mL of 1M Tris-HCl buffer at pH 7.5. Starch hydrolysis was determined from the release of glucose, measured using a glucose oxidase enzyme kit (Laborlab, Guarulhos, SP, Brazil) by way of a colorimetric reaction (Fleming, & Pegler, 1963). Sample absorbance was measured at 510 nm using a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA, USA). One unit of enzyme (U) was defined as the amount of enzyme able to produce one µmol of glucose during the reaction time. Tubes containing only starch and only enzyme were used as the controls.

The standard curve was obtained using 10, 8, 6, 4, 2, 1, 0.5, 0.25 and 0.125 mmol of glucose solution. The glucose reacted with the glucose oxidase enzyme kit and sample absorbance was measured at 510 nm in triplicate.

8.2.2. Glucose oxidase

The glucose oxidase (GO) evaluated in these experiments was a commercial enzyme from Prozyn Biosolutions® (São Paulo, Brazil). It is a yellow powder obtained as a fermentation product from *Aspergillus niger*. It has an expected molecular weight of 150 KDa, contains 16% of carbohydrate and is active in the pH range from 3.5 to 7.0 and at temperatures up to 60ºC, with optimum activity at 50°C.

GO activity was determined using the method described by Kona, Quereshi, & Pai (2001) with a few modifications: 400 µL of enzyme solution (0.3 g of dried enzyme per liter of 0.1M acetate buffer, pH 5.0 containing 0.02 $g.L^{-1}$ of sodium nitrate) was added to 400 µL of a 4 $g.L^{-1}$ glucose (for analysis degree with purity of 99.8%) solution (Synth, Brazil) and to 1.2 mL of 0.1 M acetate buffer pH 5.0. The reaction

was carried out at 50ºC and 75ºC for 30 minutes. 1.5 mL of DNS (dinitrosalicylic acid) solution was then added followed by heating at 100ºC for 5 minutes to stop the reaction. A control sample was obtained using a similar procedure, but without the addition of the GO solution. After heating, the samples were cooled and 6.5 mL of 0.1 M acetate buffer (pH 5.0) added. The absorbance was measured at 547 nm in a DU 800 spectrophotometer (Beckman Coulter®, Brea, CA, USA).

The standard curve was obtained using glucose solutions at concentrations of 0.5, 1, 2, 3, 4 and 6 $g.L^{-1}$ prepared in 0.1 M acetate buffer pH 5.0. The glucose was reacted with the DNS following the procedure described above, and absorbance measured at 547 nm in triplicate. The absorbance of the samples was converted to glucose concentration using the standard curve, and the GO activity calculated from the difference in glucose concentration between the control and the GO samples. One enzyme unit was defined as the amount of enzyme which converted 1 µg of glucose per minute. The final GO activity was calculated per gram of commercial dried enzyme.

8.2.3. Neutral protease

The neutral protease used in these experiments was a commercial metalloprotease enzyme from Prozyn Biosolutions® (São Paulo, Brazil). The enzyme is presented as a yellow powder obtained as a fermentation product from *Bacillus subtilis*. The enzyme has an expected molecular weight of 19-37 kDa, an optimum pH at 7.5 and optimum temperature at 55ºC.

The protease activity was determined using the method described by Merheb et al. (2007) with a few modifications: 200 µL of enzyme solution (0.1 g of dried enzyme per liter of 0.1 M phosphate buffer pH 7.5) was added to 400 µL of casein solution at 0.5% (w/v) (97.5% of purity, Synth, Brazil) and to 400 µL of the same buffer. The reaction was carried out at 20ºC and 55ºC for 30 minutes and 1 mL of 10% (w/v). The trichloroacetic acid (TCA) then added to stop the reaction. The samples were centrifuged at 10,000 rpm/ 5 min/ 10ºC and the absorbance measured at 275 nm in a DU 800 UV-VIS spectrophotometer (Beckman Coulter ®, Brea, CA,

USA). One unit of enzyme was defined as the amount of enzyme required to increase the absorbance at 275 nm by 0.1 unit under the assay conditions. The control samples were prepared by adding the TCA to the tubes before adding the enzyme solution, and the Δ abs_{275nm} was determined from the difference in absorbance between the sample and the control. The enzyme activity was calculated according to *Equation* 8.1.

$$U/g = \Delta Abs_{275nm} \cdot 10 \cdot \text{dilution factor} / (0.2) \qquad (\textit{Equation}\ 8.1)$$

8.2.4. High pressure homogenization of enzymes

A Panda Plus High-Pressure Homogenizer (GEA-Niro-Soavi, Parma, Italy) was used in the experiments. The equipment has a single acting intensifier pump that amplifies the hydraulic pressure up to 200 MPa.

A volume of 4 L of enzyme solution at 24ºC was homogenized under pressures of 150 and 200 MPa (amyloglucosidase and protease) and of 100 and 150 MPa (glucose oxidase). All the samples were homogenized using 3 consecutive passes, and immediately after, were cooled using a heat exchanger. The maximum pressure levels were chosen considering the operational capacity of the equipment and the level of pressure that caused a significant increment in activity of the enzymes, designated as the optimum pressure (Tribst, Augusto & Cristianini, 2012a; Tribst & Cristianini, 2012b,c). The enzyme activity was also measured after homogenization at 150 MPa (neutral protease and AMG) and 100 MPa (GO) to evaluate if the same improvement in enzyme activity observed at 200 MPa (neutral protease and AMG) and 150 MPa (GO) could be reached after HPH at lower pressures (50 MPa lower) but using multiple passes, as previously shown by Liu et al. (2009a).

After each pass, a total of 50 mL sample was collected. Unprocessed enzyme (native) was evaluated as the control (zero passes). The temperatures were measured using a needle thermometer inserted into the enzyme solution before

homogenization and in the samples collected immediately after the homogenization process.

The enzyme activities were determined immediately after homogenization at the optimum temperature (50, 55 and 65ºC for GO, protease and AMG, respectively) and at an extreme temperature (75, 20 and 80ºC for GO, protease and AMG, respectively). The extreme temperatures were chosen considering previous results that showed an improvement in enzyme activity after applying HPH (Tribst, Augusto & Cristianini, 2012; Tribst & Cristianini, 2012b,c). This evaluation is interesting since the industrial applications of many enzymes are carried out at non-optimum temperatures. The residual activity was calculated using *Equation* 8.2.

$$\text{Residual activity (\%)} = (\text{activity}_{\text{after\_HPH}} / \text{activity}_{\text{at\_optimum\_temperature\_before\_HPH}}) .100 \quad (\textit{Equation}\ 8.2)$$

8.2.5. Statistical analysis

The analysis of variance (ANOVA) was carried out to compare the effects of the different treatments, and the Tukey test used to determine the difference between them at a 5% confidence level. The statistical analyses were carried out using the STATISTICA 5.0 software (StatiSoft, Inc., Tulsa, Okla., U.S.A.). All the processes and the determinations of enzyme activity were carried out in triplicate and the results represented as the mean ± standard deviation.

8.3. Results and discussion

Table 8.1 shows the results obtained for enzyme activity prior to the HPH treatment. The maximum activities for AMG, GO and neutral protease were measured at 65, 50 and 55^{o}C, respectively, and were considered as 100% of residual activity for each enzyme.

The inlet temperature of the enzyme solutions in the homogenizer was 24ºC (room temperature) and the maximum temperature reached after homogenization was 47ºC, with a residence time < 10 s. These temperatures were not sufficient to

cause thermal denaturation of any enzymes, since the three enzymes studied have optimum temperatures above the temperature reached during the process. Therefore, 47ºC is not a denaturation temperature for GO, AMG and protease. To evaluate only the homogenization effect, the samples were cooled to 24ºC after each process and before the subsequent homogenization.

Table 8.1. Enzymes activity at different temperatures

Enzyme	Activity (optimum temperature)	Activity (non-optimum temperature)
AMG	1117750 U/g (65^{o}C)	151392 U/g (80 ^{o}C)
GO	1135313 U/g (50^{o}C)	917924 U/g (75 ^{o}C)
Neutral protease	223756 U/g (55 ^{o}C)	113292 U/g (20 ^{o}C)

Figure *8.1* demonstrates the effects of multiple HPH passes at 150 and 200 MPa on the AMG activity at 65 and 80ºC. The enzyme activity measured at 65ºC showed that HPH at 150 MPa did not improve the activity after one pass, and slightly reduced it after 2 and 3 passes through the homogenizer. To the contrary, homogenization at 200 MPa significantly increased the AMG activity after one pass (± 6%) but caused a reduction in activity after 2 and 3 passes. Considering that each pass through the homogenizer gives the enzyme a certain amount of energy, it is supposed that during the first homogenization the enzyme unfolded, resulting in increments in activity when exposed to 65ºC. After two homogenization processes, the continuing unfolding of the enzyme reduced the number of exposed active sites or caused alterations in molecular conformation, resulting in a reduction in enzyme activity.

No significant differences were observed for samples homogenized at 150 MPa and 200 MPa after 2 and 3 passes. This suggested that for AMG, a single pass through the homogenizer exercised the maximum effect on the enzyme, and subsequent homogenization was not able to change the AMG activity anymore; thus

alterations occurring after the second or third homogenization passes not being sufficient to further change the enzyme activity.

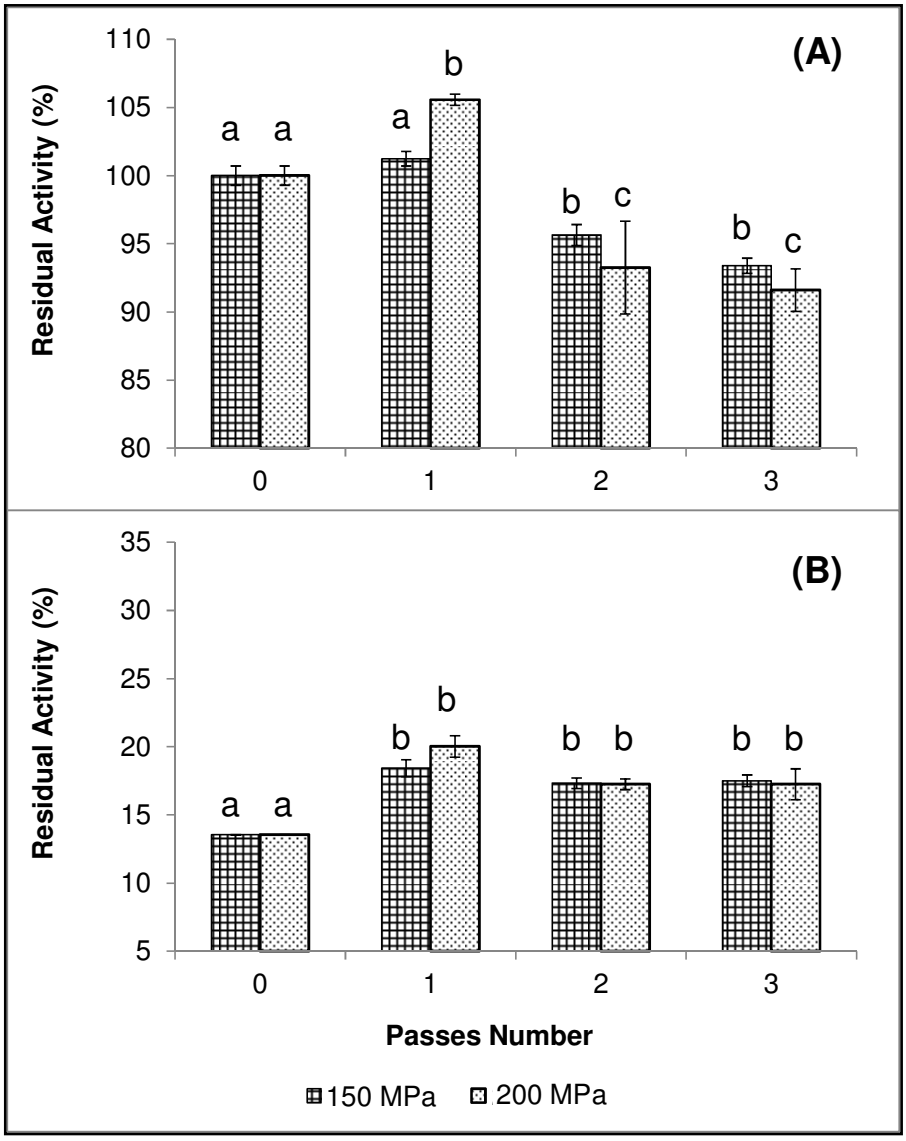

Figure 8.1. Effects of the number of sequential homogenization (passes) on amyloglucosidase activity as measured at 65 (A) and 80°C (B). Different letters mean significant difference (p<0.05) between samples evaluated at the same temperature.

An evaluation of the results revealed that the AMG was highly stable to HPH, with a maximum activity loss of 10% even after 3 passes at 200 MPa. This is important since AMG can be added to juices aiming to reduce their viscosity and

turbidity due to the starch content (Ribeiro, Henrique, Oliveira, Macedo, & Fleuri, 2010), and HPH is being proposed as an alternative for juice processing to improve the sensory quality of the juice (Tribst et al., 2011). Therefore, the enzyme can be added to the juice before processing, and the residence time for enzyme activity can occur just after homogenization. This allows for the use of the heating (consequence of intense shear) to help the sample reach the desired temperature for AMG activity, saving time and reducing heating costs.

The AMG activity measured at 80ºC showed that HPH was able to improve the AMG activity after one pass at 150 and 200 MPa ($p<0.05$). No significant differences were observed after HPH with 1, 2 or 3 passes for either of the pressures ($p > 0.05$), corroborating the hypothesis that the maximum HPH changes in AMG structure were reached after a single HPH process under those conditions. The differences in activity improvement after HPH when the AMG activity was measured at 65 and 80ºC can be explained considering that the enzyme conformation is dependent on both the homogenization pressure applied and the temperature used to determine the activity. Therefore, when a temperature of 80ºC was used to determine the activity, the changes caused by homogenization at 150 MPa were sufficient to cause significant improvement in enzyme activity. The improvement in AMG activity at 80ºC can be advantageous when the enzyme is applied in starch saccharification, a process in which the starch is previously gelatinized and then cooled down to allow the AMG to act (Mamo & Gessesse, 1999). Therefore, an AMG that is active at higher temperatures allows the starch saccharification process to begin at higher temperatures than commonly applied in the traditional process (Mamo & Gessesse, 1999), resulting in time and energy savings.

Figure 8.2 shows the effects of multiple HPH passes at 100 and 150 MPa on GO activity at 50 and 75ºC. The enzyme activity measured at 50ºC showed that one pass at 100 MPa slightly reduced the activity of enzyme, and no differences between the native and homogenized samples (one pass) were observed after 2 and 3 passes at the same pressure. The maximum activity loss observed was about 10%, indicating that the enzyme was resistant to homogenization at this pressure. This is interesting since HPH can be applied as an alternative method in food processing, including food

with glucose oxidase added to improve its sensory characteristics during the shelf life, e.g. pasteurized juice with added GO to prevent oxidation (Bankar, Bule, Singhal & Ananthanarayan, 2009). To the contrary, 150 MPa caused a gradual loos in activity of GO at 50ºC, being significant after 2 passes through the homogenizer ($p<0.05$). These results may indicate that HPH at 150 MPa has an additional effect on the GO structure for each homogenization pass.

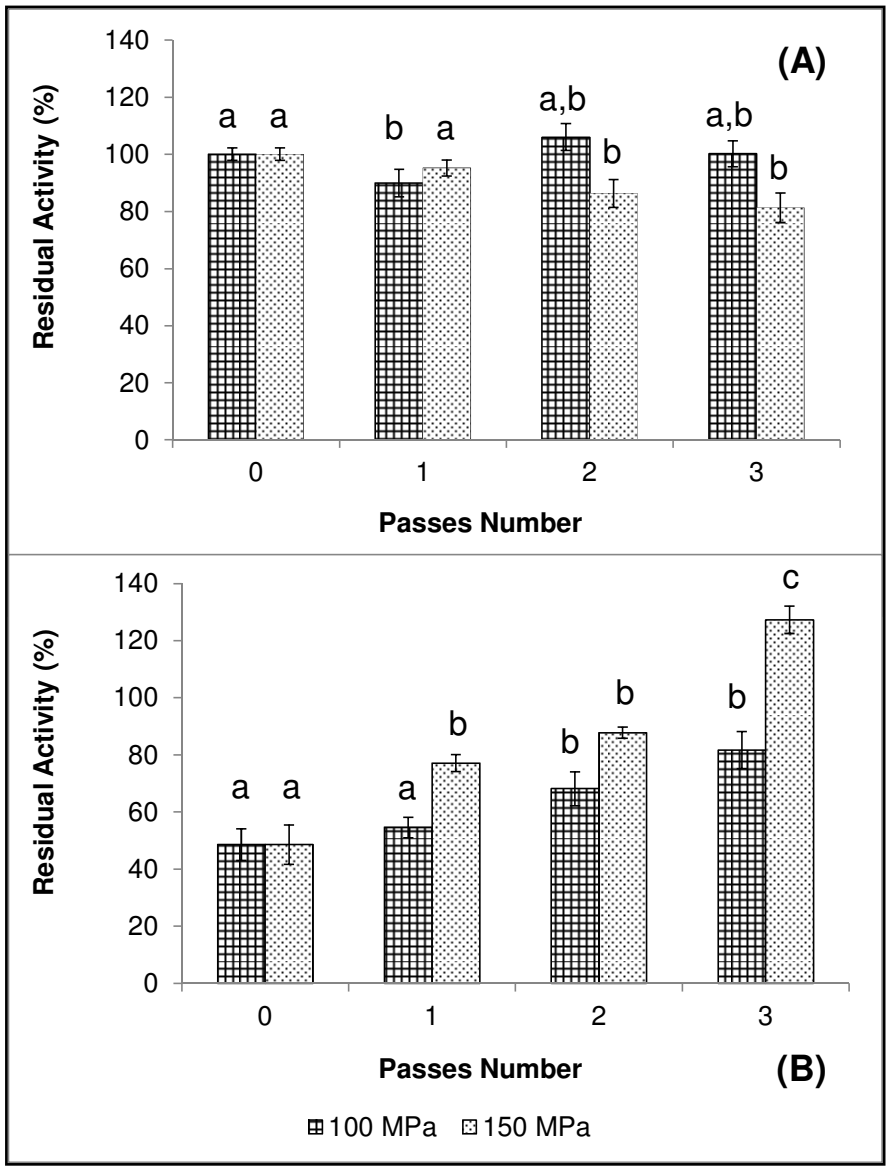

Figure 8.2. Effects of the number of sequential homogenization (passes) on glucose oxidase activity as measured at 50 (A) and 75°C (B). Different letters mean significant difference ($p<0.05$) between samples evaluated at the same temperature.

Multiple HPH passes promoted an intense activity change in GO at 75ºC. For both pressures evaluated (100 and 150 MPa), a gradual increase in enzyme activity was observed, which was significant after 2 passes at 100 MPa (increase of around 30%) and after 1 pass at 150 MPa (increase of around 60%). Also, one pass at 150 MPa and two at 100 MPa caused the same improvement in GO activity at 75ºC.

It is interesting to highlight that 3 passes of HPH at 150 MPa was able to improve the activity of GO at 75ºC by 2.5 times, resulting in greater activity than the native enzyme at its optimum temperature (50ºC). Therefore the optimum temperature shifted from 50 to 75ºC after three homogenization passes.

Previous results also indicated that HPH changed the optimum temperature for protease activity (Tribst, Augusto & Cristianini, 2012a), showing that HPH may cause changes in enzymes that directly affect the exposure of active sites and improve the lock and key mechanism between enzyme and substrate at non optimum temperatures, considering the new molecular conformation due to spatial enzyme alterations caused by homogenization. This is especially useful when GO must be active at high temperatures or the enzyme needs to remain active after a thermal process. Examples of such applications are the addition of GO to pasteurized juice to prevent oxidation during the shelf life (Bankar et al., 2009).

Figure 8.3 shows the effects of multiple HPH passes at 150 and 200 MPa on neutral protease activity at 20 and 55ºC. The enzyme activity measured at 55ºC indicated that HPH reduced the protease activity by about 60% at 150 and 200 MPa and similar results were obtained after one, two or three passes. When the activity was measured at 20ºC, HPH at 150 MPa did not alter the enzyme activity, even after 3 passes. To the contrary, a significant increase (around 20%) in protease activity at 20ºC was detected after homogenization at 200 MPa (one or two passes) and a slight reduction in activity was observed after 3 passes. Therefore, the results suggest that the main effects of HPH on neutral protease occur during the first homogenization pass, with minimum additional changes during the second and third passes.

The results obtained for each enzyme evaluated illustrate that the effect of multiple passes was dependent on the pressure level applied, the temperature the activity was measured at and the enzyme evaluated. Depending on these factors, the

process caused an increase, reduction or maintenance of the enzyme activity. Previous results also demonstrated that pectin methylesterase activity was not affected by five HPH passes at 100 MPa (Welti-Chanes, Ochoa-Velasco, & Guerrero-Beltrán, 2009) or at 170 MPa (Lacroix, Fliss, & Makhlouf, 2005), while polyphenol oxidase from mushrooms and pears showed a significant improvement in activity after three HPH passes at 150 and 160 MPa, respectively (Liu et al., 2009a,b).

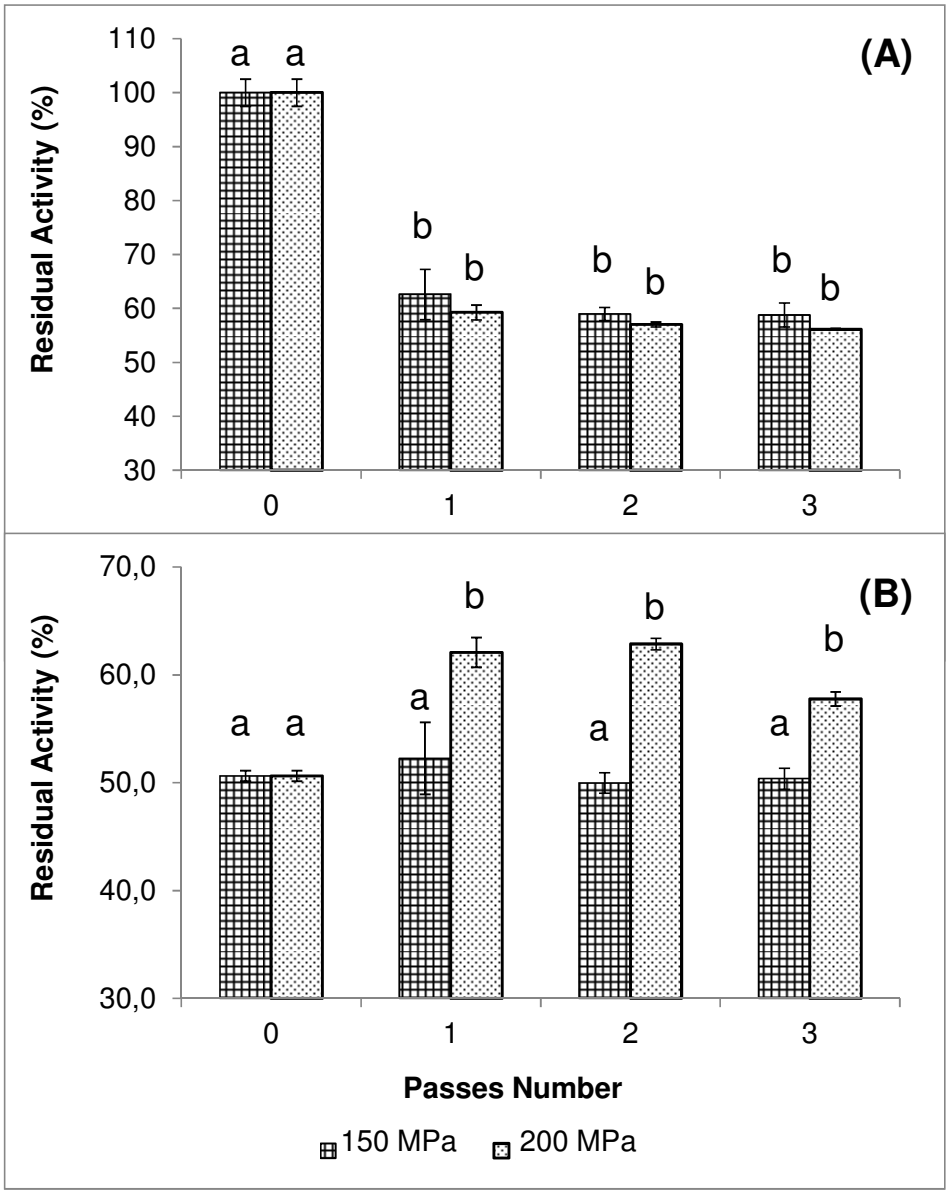

Figure 8.3. Effects of the number of sequential homogenization (passes) on neutral protease activity as measured at 55 (A) and 20°C (B). Different letters mean significant difference ($p<0.05$) between samples evaluated at the same temperature.

HPH involves considerable mechanical forces (Keerati-U-Rai & Corredig, 2009), which results in an intense and abrupt energy input into the homogenized samples. This energy is large enough to alter the α-helix, β-sheet and β-turn structures of the enzymes (Liu et al., 2009b, 2010), modifying the enzyme structure due to changes in molecular interactions and linkages (Liu et al., 2009b, 2010).

Previous research assessed the effect of HPH on different protein sources. The process was able (Gárcia-Juliá et al., 2008; Luo et al., 2010; Dong et al., 2011; Poliseli-Scopel et al., 2012; Yuan et al., 2012) or not (Bouauina et al., 2006) to change the protein conformation. The process energy is considered large enough to disrupt the tertiary and quaternary structures of most globular proteins (Subirade et al., 1998), which may induce rearrangements and aggregation (Keerati-U-Rai & Corredig, 2009).

The effects reported with respect to protein structure were denaturation, unfolding or dissociation (Dong et al., 2011), resulting in an increase in the area of protein exposed (Dong et al., 2011) and even broken protein (Luo et al., 2010). The consequences observed were a reduction in protein molecular weight (Dong et al., 2011) and an increment in the reducing power and that of hydroxyl radical scavenging (Dong et al, 2011). The formation of new secondary bonds was also reported (Luo et al., 2010) and an increment in hydrophobic interactions (Gárcia-Juliá et al., 2008, Luo et al., 2010, Yuan et al., 2012) resulting in a build-up of protein aggregates (Gárcia-Juliá et al., 2008, Keerati-U-Rai & Corredig, 2009; Luo et al., 2010; Yuan et al., 2012).

These aggregates can be more soluble than the native protein (Luo et al., 2010; Yuan et al., 2012), which is mainly related to the soluble aggregates that attach themselves easily to the interface, forming a thicker adsorbed layer (Luo et al., 2010).The destruction of the tight structure of the proteins and of the insoluble aggregates was also observed. Thus, HPH alters the proteins, and consequently enzymes, in many ways.

No previous investigations have explained the effect of multiple HPH passes on the structure of enzymes or proteins. For polysaccharides, multiple HPH passes induced continuous depolymerization, broken chains and a reduction in molecular

size. However, the main effects occurred in the first steps (Lagoueyete & Paquin, 1998; Kivelä et al., 2010; Villay et al., 2012) and additional passes had less and less impact on the average molecular weight (Lagoueyete & Paquin, 1998) and viscosity (Villay et al., 2012) (i.e., the effect of the multiple passes is asymptotic).

This can be explained by the reduction in molecular size to the minimum for each homogenization pressure, which occurs after the first pass through the homogenizer for the majority of polysaccharides. Thus additional mechanical effects cannot further reduce the molecular size (Lagoueyete & Paquin, 1998; Villay et al., 2012). On the other hand, after the polymer chains are open, they become more susceptible to homogenization-induced degradation, since a greater number of bonds are directly accessible to the mechanical stress (Lagoueyete & Paquin, 1998). These effects are more dependent on differences in the polysaccharide structures and conformation (linear, branched) than in polymer charge or molar mass (Villay et al., 2012). The process results in ordered-disordered conformational transition (by opening the molecule) and polymer degradation. The opening of the molecule occurs first, followed by polymer degradation due to mechanical stress (Lagoueyete & Paquin, 1998). Thus many of the effects of HPH were similar for proteins and polysaccharides.

Lander et al. (2002) discussed that the fluid shear during HPH is the dominant mechanism for polysaccharide breakage. Since the relative importance of these phenomena is related to the range in sizes between the sample (molecule, cell, drop,…) and the turbulent eddies (Inning et al., 2011), similar behavior can be expected for proteins.

At each pass through the homogenizer, the sample is submitted to the same magnitude of shear stress, and consequently to the same mechanical energy. At each pass, only those molecular bonds whose energy is smaller than the associated mechanical energy, are broken. This explains the asymptotic behavior observed in polysaccharides (Lagoueye & Paquim, 1998; Lander et al., 2000; Ronkarz et al., 2010; Harte and Venegas, 2010) and also those expected for protein unfolding.

When an enzyme unfolds, both activation and inactivation are expected. This conformational change can expose the active site and increase its activity or prevent its contact with the substrate, reducing enzyme activity.

In fact this explains the results obtained, since the amyloglucosidase activity first increased and then decreased (*Figure 8.1a*), the glucose oxidase activity just increased (*Figure 8.2b*) and the neutral protease activity just decreased (*Figure 8.3a*). Therefore, although the effect of multiple passes on the unfolded protein is expected to be asymptotic, the effects on enzyme activity do not necessarily follow that behavior.

Considering the results obtained in the present research, some of the conditions studied resulted in no change in enzyme activity even after three passes, possibly indicating that the energy provided by the multiple passes was not able to change the enzyme molecules sufficiently to alter the activity of GO at 50ºC or of neutral protease at 20ºC. Also, at the optimum temperatures, only AMG showed a slight improvement in activity and only after one pass at 200 MPa. This may indicate that the native enzyme configuration is the best one to react at the optimum temperature, since this was chosen based on the enzymatic reaction of the native form.

To the contrary, improvements in activity at non-optimum temperatures were observed for all enzymes, and the maximum enzyme activity increase occurred after only one pass for AMG and neutral protease and after three passes for glucose oxidase. This possibly indicates that HPH was able to alter part of the structure of the AMG and protease during the first pass, and the subsequent energy gain (during the second and third passes) was not sufficient to significantly change the enzyme configuration (suggesting that the maximum molecular change occurred during the first pass). In contrast, each energy gain due to a single homogenization pass modified the glucose oxidase configuration, suggesting that the protein chains were open as from the first pass, becoming more susceptible to homogenization-induced changes due to the increase in bonds directly exposed to the mechanical stress, similarly to that observed with xanthan gum (Lagoueyete & Paquin, 1998).

The results highlighted the fact that the main gain in enzyme activity occurred at non optimum temperatures, suggesting that HPH changes the enzyme spatial configuration (enhancing the exposure of active sites under non optimum conditions) or improves enzyme stability due to strong linkages or aggregates formed after high pressure homogenization.

8.4. Conclusions

HPH affected the activity of amyloglucosidase, glucose oxidase and neutral protease, particularly with respect to improving it at non-optimum temperatures. For amyloglucosidase and neutral protease, the main effects of homogenization were observed after only one pass, indicating that the energy gain of the enzyme under this condition was sufficient to effect the maximum molecular changes caused by homogenization. To the contrary, the continuous improvement in the activity of glucose oxidase can be attributed to the additional molecular change caused by each homogenization pass. Therefore, HPH can be applied to improve enzyme activity and the efficacy of multiple passes is dependent on the kind of enzyme.

Acknowledgments

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Conclusões Gerais

A homogeneização à alta pressão (HAP) é um processo não térmico desenvolvido para garantir a estabidade microbiológica de alimentos fluidos, como leite e sucos de frutas. Atualmente, os efeitos do processo sobre micro-organismos diversos já foram bem estabelecidos e o processo foi caracterizado como sendo similar a pasteurização térmica, capaz de inativar bolores, leveduras e bactérias não esporuladas. Estudos mais recentes avaliaram o processo como uma operação unitária capaz de alterar os constituintes dos alimentos processados, incluindo proteínas, lipídeos, polissacarídeos e enzimas.

Para produtos de origem vegetal, a avaliação do efeito do processo sobre a polifenoloxidase, uma enzima reconhecidamente indesejável para alimentos por causar escurecimento dos tecidos em função da oxidação de compostos fenólicos e sua polimrização, mostrou que a HAP é capaz de aumentar atividade da enzima em até 80%. Apesar disto representar uma dificuldade para aplicação da tecnologia em termos de produção de sucos, estes resultados indicaram que a HAP poderia ser utilizada como um processo de ativação de enzimas. A partir destes dados se estabeleceu o objetivo desta pesquisa, que foi avaliar o efeito da HAP em enzimas de interesse comercial.

A avaliação global dos resultados obtidos mostrou ser impossível estabelecer uma regra para o efeito do processo sobre as enzimas, uma vez que os resultados foram diferentes em função de cada enzima, das condições estudadas de processo (pH da solução, temperatura de processo e pressão aplicada) e também das condições utilizadas na medida de atividade (pH e temperatura). Desta forma, algumas conclusões foram obtidas individualmente para cada enzima.

A α-amilase de *A. niger* foi, dentro do grupo de enzimas estudadas, aquela que apresentou maior estabilidade ao processo. A HAP não foi capaz de alterar a atividade da enzima em pH e temperaturas de atividade ótimos e não ótimos, ainda quando o processo foi realizado na ausência de cálcio no meio, que tem a função de estabilizar a molécula da enzima. Assim, para esta enzima conclui-se que a energia fornecida durante a homogeneização até 150 MPa foi insuficiente para promover a quebra de ligações e interações importantes para a estabilidade da molécula ou que,

caso elas tenham sido rompidas, a enzima logo após o processamento conseguiu recobrar sua configuração original.

A protease neutra de *B. subtilis* foi sensível ao processo, sendo observada mudança na enzima após a HAP nas três temperaturas em que a sua atividade foi medida. Para todas as temperaturas, a alteração da atividade ocorreu de forma contínua com o incremento da pressão de homogeneização, indicando que o aumento de energia fornecida às moléculas a cada aumento de pressão foi suficiente para promover rompimento de ligações/ interações que garantiam a manutenção da estrutura da enzima. Estas alterações causadas a 200 MPa resultaram em perda de atividade na condição ótima, mas aumento de atividade da protease a 20ºC, o que é industrialmente interessante para processos onde o aquecimento da proteína a ser hidrolisada só é realizado em função da temperatura ótima da enzima.

A amiloglicosidase de *A. niger* também foi alterada pela homogeneização à alta pressão e os resultados foram dependentes do pH de processo. Para esta enzima, não foi observada redução da atividade com a aplicação da homogeneização em diferentes pressões. Por outro lado, os ganhos significativos de atividade observados para a enzima, especialmente a altas temperaturas (80ºC), demonstraram que as alterações causadas pela homogeneização possivelmente aumentaram a exposição dos sítios ativos da enzima a alta temperatura ou mantiveram a estrutura mais termicamente estável. Este ganho é especialmente interessante considerando-se que a principal aplicação da amiloglicosidase é realizada no processo de sacarificação do amido. Neste processo, as temperaturas iniciais das soluções são bem elevadas e normalmente precisam ser reduzidas para a aplicação da amiloglicosidase.

A β-galactosidase de *K. lactis* foi, entre as enzimas estudadas, a que apresentou os piores resultados após a HAP, com redução de até 30% de atividade após processamento a 150 MPa para quase todas as condições avaliadas. A única condição na qual a enzima se manteve estável foi quando processada em seu pH ótimo e a baixas temperaturas, indicando que o pH e a força iônica do meio tem um papel importante na manutenção da estabilidade desta enzima.

Por fim, resultados obtidos para a glicose oxidase de *A. niger* mostraram que a enzima foi bastante modificada pelo HAP, atingindo diferentes atividades em função do pH e pressão de homogeneização e temperatura na qual a atividade foi mensurada. De uma forma geral, foi observado que a enzima apresentou mudanças contínuas com o incremento da pressão aplicada no processo, e, para a maioria das condições nas quais foram observadas reduções de atividade, isso aconteceu após HAP a 50 ou 100 MPa. Por outro lado, o processamento a 150 MPa resultou em recuperação ou aumento de atividade da enzima. A intensa modificação observada para a glicose oxidase pode ser atribuída à complexa estrutura da enzima, que é mantida por muitas interações fracas que são possivelmente rompidas em função do nível de pressão utilizada nos diferentes processos de homogeneização. Os melhores efeitos do processo foram observados quando a atividade da enzima foi medida a 75ºC, indicando que, conforme observado para a amiloglicosidase, o processamento resultou em aumento da exposição dos sítios ativos da enzima ou promoveu a estabilização das mesmas em alta temperatura. Este resultado é interessante quando se deseja aplicar a glicose-oxidase a uma temperatura superior a de sua atividade ótima.

De uma forma geral também foi possível concluir que a HAP em temperaturas elevadas afeta negativamente a atividade das enzimas, uma vez que a realização da homogeneização em temperaturas próximas as condições ótimas de atividade das enzimas (50-60ºC) resultou, para a maioria das enzimas avaliadas, em perda de atividade parcial ou total. O binômio tempo/temperatura atingido para cada enzima foi certamente inferior ao necessário para inativação térmica das enzimas estudadas, portanto, conclui-se que a HAP torna a enzima mais susceptível a desnaturação. Uma exceção a esta conclusão foi observada para a α-amilase, que se manteve estável mesmo quando submetida à HAP à temperatura inicial de 65ºC.

A reversibilidade do efeito da HAP, avaliada indiretamente pela atividade das enzimas nativas e homogeneizadas após um período de repouso, ocorreu apenas para algumas amostras de amiloglicosidase e protease e foi dependente das condições de processo. Para as demais enzimas e condições avaliadas, as atividades das enzimas nativas e homogeneizadas foram diferentes após a

estocagem, a partir do que se conclui que a enzima homogeneizada atingiu uma configuração estável ou após o processo ocorreu uma nova reacomodação da molécula, atingindo uma terceira configuração. Vale destacar novamente os resultados obtidos para a glicose oxidase, cujo aumento de atividade após um dia de estocagem ficou entre 100 e 400% quando comparada à enzima nativa estocada nas mesmas condições. Este representou um dos maiores ganhos obtidos pelo processo de HAP estudado, uma vez que uma das aplicações da enzima é a adição em alimentos embalados para consumo de oxigênio, evitando assim oxidação de pigmentos, compostos aromáticos e vitaminas.

Para as enzimas que apresentaram ganhos de atividade ou estabilidade, foi realizado um novo estudo do efeito da HAP considerando a aplicação de múltiplos processos. A partir dos resultados foi possível concluir que, para a maioria das enzimas e condições avaliadas, o uso de processamentos sequenciais resultava em não ateração da atividade enzimática ou na redução da mesma. Este resultado pode ser explicado pelo fato de que cada processo fornece o mesmo nível de energia para as moléculas, o que consequentemente resulta em baixa habilidade de modificação das estruturas já modificadas no processamento inicial. Resultados obtidos para a glicose oxidase com atividade medida a 75ºC, por outro lado, indicaram um aumento da atividade de forma contínua. Assim, concluiu-se que, quando a molécula é muito instável e formada por interações e ligações fracas, o efeito dos processos sequenciais pode ser contínuo.

A avaliação global dos resultados permite concluir que a HAP é um processo promissor para modificação de enzimas, especialmente quando se deseja aumentar sua atividade em condições não ótimas. Entretanto, foi observado que não é possível fazer uma generalização dos efeitos, sendo necessária a avaliação dos resultados obtidos para cada enzima nas condições em que se deseja obter o aumento de atividade. Alguns resultados obtidos no presente trabalho apresentam direta aplicação industrial, podendo servir de guia para modificação de enzimas com aumento de atividade em temperaturas não ótimas de atividade (glicose oxidase, amiloglicosidase e protease), ou temperatura ótima de atividade (amiloglicosidase)

ou, ainda, aumento da estabilidade frente a estocagem em solução aquosa (glicose oxidase).

A Tabela 9.1 sumariza os resultados obtidos de forma a permitir uma avaliação uma avaliação comparativa dos dados.

Sugestões para trabalhos futuros

Os resultados obtidos nesta tese aumentam substancialmente as informações disponíveis sobre o efeito da HAP em enzimas além de ter direta aplicação industrial. A partir dos dados obtidos, sugere-se, para trabalhos futuros a continuidade dos estudos do processo de HAP sobre outras enzimas de importância comercial, especialmente aquelas que apresentam estruturas complexas que, aparentemente, apresentam-se mais susceptíveis às alterações causadas pela HAP. Também é importante avaliar como o efeito da homogeneização sobre as enzimas pode ser afetado por diferentes concentrações das soluções enzimáticas.

Além disso, sugere-se que novos trabalhos sejam realizados com enzimas com alto grau de pureza possibilitando uma análise biofísica da molécula após a HAP para determinar os efeitos do processo nas estruturas da enzima e, consequentemente, estabelecer o papel de cada estrutura e da conformação final das enzimas na estabilidade e atividade das mesmas.

Tabela 9.1. Efeito da homogeneização à alta pressão na atividade e estabilidade das enzimas comerciais avaliadas

Enzima	Efeito da HAP se atividade medida em condições ótimas (pH, T)	Efeito da HAP se atividade medida em pH ótimo e T não ótima	Efeito da HAP se atividade medida em pH e T não ótimas	Efeito da HAP a alta T na atividade da enzima	Estabilidade após estocagem refrigerada	Observações
α-amilase	Atividade se manteve em $P_H \leq 150$ MPa	Não observada modificações na atividade em $P_H \leq 150$ MPa	----	Não modificou a atividade após $P_H = 150$ MPa, se $T_{inicial} = 65$ºC	Mantida entre enzima nativa e homogeneizada ($P_H = 150$ MPa).	O cálcio não afetou a estabilidade da enzima durante a HAP
PN	Reduziu gradualmente até 40% em $P_H \leq 200$ MPa	Atividade reduziu quando medida a 70ºC ($P_H \geq 50$ MPa) e aumentou quando atividade medida a 20ºC ($P_H = 200$ MPa)	----	Atividade reduziu (~50%) após $P_H = 200$ MPa, se $T_{inicial} = 60$ºC	Mantida se atividade medida a 20ºC e reduzida se a atividade medida a 55ºC ($P_H = 200$ MPa)	Sem modificações na absorção de luz UV pela enzima após HAP em $P_H \leq 200$ MPa
AMG	Aumentou gradualmente (entre 5-8%) em $P_H \leq 100$ MPa	Atividade manteve-se em $P_H \leq 200$ MPa (35ºC) ou aumentou gradualmente ~30% até $P_H = 100$ MPa (80ºC)	Atividade da enzima modificou (aumentou) quando a mesma foi medida a 65 e 80ºC.	Atividade reduziu (~100%) após $P_H = 150$ MPa, se $T_{inicial} = 65$ºC	Variou em função do pH e T. Para a maioria dos ensaios, atividade se manteve ($P_H = 200$ MPa).	----
β-GL	Atividade se manteve em $P_H \leq 150$ MPa	Atividade reduziu (~20%) após HAP a 150 MPa.	Atividade reduziu quando $P_H = 150$ MPa, em pH 6,4 e 8,0 e T= 15 ou 45ºC	Atividade reduziu (+ de 50%) após $P_H = 150$ MPa, se $T_{inicial} = 20$ºC	Atividade manteve estável apenas após HAP ≤ 150 MPa, quando pH= 7,0	Absorção de luz UV foi modificada quando enzima foi homogeneizada em pH 6,4 e 8,0
GO	Atividade reduziu até $P_H = 50$ MPa e foi recobrada em $P_H \geq 100$ MPa	Atividade reduziu (~30%) após $P_H \leq 150$ MPa (15ºC). A 75ºC, atividade reduziu até 50 MPa e foi recobrada em $P_H \geq 100$ MPa.	Atividade caiu ou se manteve quando medida a 15ºC e aumentou quando medida a 75ºC	Não modificou a atividade da enzima ($P_H \leq 150$ MPa), se $T_{inicial} = 50$ºC	Atividade se manteve ou aumentou (entre 100 e 400%)	Atividade após 24h da enzima homogeneizada a 50ºC reduziu quando comparada a enzima homogeneizada a T ambiente.

PN- protease neutra; AMG – amiloglicosidase; β-GL - β-galactosidase; GO – glicose oxidase; T - temperatura

Apêndice I

Esquema de operação do homogeneizador a alta pressão