รายงานวิจัยฉบับสมบูรณ์

โครงการ การวิเคราะห์เชิงความร้อนของอันตรกิริยาพลาสม่าโปรตีน: ศักยภาพการประยุกต์สำหรับการวินิจฉัยมะเร็งเชิงวิเคราะห์ตัวบ่งชี้ทางชีวภาพ

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พฤษภาคม พ.ศ. 2563

สัญญาเลขที่ RSA6080008

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยสงขลานครินทร์

กิตติกรรมประกาศ

The investigator is grateful to Thailand Research Fund as well as Prince of Songkla University for the co-funding research grant. Special thanks also go to PSU Scientific Equipment Center, and Drug Delivery System Excellent Center, Faculty of Pharmaceutical Sciences for Lab facilities.

รหัสโครงการ: RSA6080008

ชื่อโครงการ: การวิเคราะห์เชิงความร้อนของอันตรกิริยาพลาสม่าโปรตีน: ศักยภาพการประยุกต์สำหรับ การวินิจฉัยมะเร็งเชิงวิเคราะห์ตัวบ่งชี้ทางชีวภาพ

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บทคัดย่อ

การลุกลามของมะเร็งทำให้กิจกรรมของเอนไซม์โปรติเอสสูง ร่างกายจึงสร้างแปปไทด์ที่ผิดปกติในปริมาณมากอย่างมีนัยสำคัญซึ่งแปปไทด์เหล่านี้อาจเป็นตัวบ่งชี้ทางชีวภาพได้ แปปไทด์บ่งชี้ทางชีวภาพนี้สามารถจับกับอัลบูมิน (เรียกแปปไทด์ว่า albuminone) *และมีผลต่อสภาพความคงตัวต่อความร้อนของอัลบูมินในเลือด งานวิจัยนี้ มุ่งศึกษาความคงตัวต่อความร้อนของโปรตีนในพลาสม่า โดยมุ่งเน้นอัลบูมินจากโรคมะเร็งเต้านมเทียบกับสภาวะปกติโดย* Differential Scanning Calorimetry (DSC) *เพื่อค้นหาทางเลือกที่มีศักยภาพในการประยุกต์สำหรับการวินิจฉัยมะเร็ง ทำการเก็บตัวอย่างเลือดจากอาสาสมัครผู้ป่วยหญิงที่วินิจฉัยแล้วว่าเป็นมะเร็งเต้านม 11 ราย และจากอาสาสมัครหญิงสุขภาพสมบูรณ์ 50 คน นำเลือดมาปั่นแยกพลาสม่าและสกัดเอาอัลบูมินจากพลาสม่าเพื่อวิเคราะห์ความคงตัวของโปรตีนด้วย* DSC *สแกนอุณหภูมิระหว่าง 37 และ 90* °C *ในอัตรา 5 องศาต่อนาที* DSC thermograms *ของพลาสม่าปกติทุก ๆตัวอย่างมีอัตลักษณ์เฉพาะแบบเดียวกัน ทำให้สามารถแยกแยะสัญญาณการสลายตัวด้วยความร้อนของอัลบูมินจาก* thermogram *ได้ และเมื่อเทียบกับพลาสม่ามะเร็งเต้านมก็สามารถบ่งชี้การเปลี่ยนแปลงของสัญญาณดังกล่าวได้ พบว่าอุณหภูมิที่อัลบูมินสลายตัวด้วยความร้อนเพิ่มขึ้นเมื่อมะเร็งลุกลามมากขึ้นซึ่งแสดงนัยของการเพิ่มขึ้นของ* albuminone/*แปปไทด์ผิดปกติที่ผลิตจากการคุกคามของมะเร็ง ทำการวิเคราะห์พลังงานจากการสลายตัวของอัลบูมินด้วยการประมาณตรรกยะของแบบจำลอง* Lumry-Eyring *ใน* thermogram *พลาสม่าของอาสาสมัครหญิงปกติได้พลังงานกระตุ้นการสลายตัวของอัลบูมินเท่ากับ* 132.68±14.21 Kcal/mole *ระดับพลังงานนี้ ลดลงเมื่อมะเร็งมีการลุกลามมากขึ้นจนมีค่าเท่ากับ* 46.76 ± 8.42 Kcal/mole *ในมะเร็จระยะสุดท้ายโดยลดลงอย่างเห็นได้ชัดระหว่างการรุกลามของมะเร็งในระยะที่ 2 ดังนั้น ค่าพลังงานกระตุ้นการสลายตัวของอัลบูมินอาจจะเป็นพารามิเตอร์ในการวินิฉัยและพยากรณ์โรคมะเร็งเต้านมซึ่งวิธีนี้มีข้อได้เปรียบ ได้แก่ ง่าย รวดเร็ว และราคาถูกเมื่อเทียบกับเทคนิคอื่น ๆ อย่างไรก็ตาม วิธีนี้ก็ยังคงไวไม่พอที่จะตรวจจับมะเร็งในระยะแรก ๆได้ จึงแนะนำให้เป็นการคัดกรองเบื้องต้นที่อาจดำเนินการควบคู่ไปกับเทคนิคทางแมส-สเปกโตรมิตรี*

คำสืบค้น: Differential scanning calorimetry; breast cancer; Lumry-Eyring model; human albumin; albuminome

Project code: ***RSA6080008***
Project title: Calorimetric analysis of plasma protein interactions: Potential application to the cancerous biomarker analysis

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Project period: May 30, 2017 – May 29, 2020

Abstract

Cancerous invasion causes increased protease activity of the host yielding unusually significant amount of peptide metabolites which may serve as biomarkers. These peptide biomarkers could become albuminone and modulate stability of plasma albumin via binding interaction. The study aimed at the investigation of thermal stability of human plasma proteins focusing on albumin with breast cancer in various stages *versus* normal ones by means of Differential Scanning Calorimetry (DSC) so as to find the potential cancer diagnosis alternative. Blood plasma was drawn from eleven female breast cancer patients and 50 healthy volunteers of homogeneous demographics. Plasma samples and their albumin-enriched fractions were subjected to DSC scanning between 37 and 90 ^{o}C at 5 ^{o}C/min rate. For normal blood plasma, a characteristic signature of DSC tracing was observed. And, the deconvolution of DSC thermograms revealed the recognition of thermal transition of albumin. It was found that denaturing temperature of albumin increased with increasing breast cancer staging which implied the increase in albuminome/peptide abundance produced by cancerous invasion. The analysis of albumin denaturing energetics based on rational approximation of simple Lumry-Eyring model demonstrated that thermal transition of healthy albumin attained energetic level expressed as apparent activation energy of 132.68±14.21 Kcal/mole. The energy decreased with increasing degree of cancerous invasion and leveled off at 46.76 ± 8.42 Kcal/mole in the very last stages. And, it markedly decreased where the disease was in stage II. Thus, it was proposed that the apparent activation energy may be used as a parameter to diagnose and assess the prognosis of breast cancer with some advantages including simpler, faster and cheaper compared to other available techniques. Nevertheless, it was not sensitive enough to detect breast cancer in early stage. Use as the purpose of screening was recommended. It was suggested that MS-based techniques should be done in addition to DSC. While DSC provided a signature thermogram indicative of a disease state, MS gave the specific details of cancerous albuminomes. They were mutually complementary in the diagnosis.

Keywords: Differential scanning calorimetry; breast cancer; Lumry-Eyring model; human albumin; albuminome

เนื้อหางานวิจัย

<u>*บทนำ*</u>

Introduction

Cancers have been the major cause of mortality. In 2008 for instance, 7.6 million people were dead due to cancers worldwide calculating as high as 13% global mortality. In 2011, 61,082 Thai people died of cancerous diseases which was ranked number one among the causes of annual mortality in Thailand, and breast cancer has been the major female cancerous invasion (National Cancer Institute, 2013). To date, no single tumor marker for diagnosis was recognized. It is because the tumor invasion always involves with many pathways of unusual protein/peptide metabolism. And, the marker targets in blood reflective of cancer in early stage, if present, are in extremely low levels (Anderson and Anderson, 2002). Cancerous progression, invasion, and metastasis always associate with protein degradation catalyzed by proteases (Koblinski et al, 2000). The increased enzyme activities cause various low molecular weight protein substrate degradation peptides circulating in blood in significant total amount. They afford a potentially rich pool for the disease related biomarker detection (Petricoin et al, 2006).

Differential scanning calorimetry (DSC) has been introduced to the field of proteomic study (5-6). It monitors the heat changes associated with the thermally induced denaturation. DSC analysis of normal blood plasma yields a unique tracing called thermogram that becomes a thermal signature of denaturing transition of plasma proteins (Garbett et al, 2009). Even though human blood plasma contains more than 3,000 proteins and peptides, only few of them, including albumin, IgG, fibrinogen, and transferrin, make up the majority of the mass. These few most abundant proteins determine the DSC thermogram as Garbett at al. (2008) demonstrated that the summation of weighed thermograms of the individual purified proteins accurately reproduced the features of average healthy plasma one. It has been further found that the characteristic thermogram altered due to the disease state. It is because pathologies and disorders trigger the change of plasma proteins composition which distorted thermogram, and the abnormality due to disease could modulate thermal stability of proteins (Vega et al, 2015). Thus, monitoring thermogram of blood plasma may lead to the disease diagnosis. A number of investigators reported that the changes of DSC calorimetric parameters namely denaturing transition temperatures, heat capacities, and enthalpies derived from blood plasma thermograms may aid the diagnosis of several diseases such as cervical cancer (Garbett et al, 2009), colorectal cancer (Todinova et al, 2012), and breast cancer (Garbett et al, 2009; Todinova et al, 20129; Zapf et al, 2011). Unfortunately, the changes of plasma proteins composition detectable by DSC always occur as consequence of common pathologies such as inflammations. While, the diagnosis requires the recognition of disease-related peptide "*biomarkers*", if present, which present at such low levels that DSC cannot detect. But, monitoring the thermal stability modulation of high abundant plasma proteins may be possible as it was observed that the DSC peak of abundant proteins in blood plasma of breast cancer patients deviated to higher temperature due to the disease state (Zapf et al, 2011).

Among plasma proteins, albumin plays a major binding partner for ligands. It has been apparent that several small molecule peptides, regardless of their chemical structures, bind with albumin (Fasano et al, 2005). For instance, 141 peptides associated with albumin were found in a commercial albumin product (Gay et al, 2010) whereas 125 albumin-interacting proteins and 14 highly-connected clusters were identified in blood plasma of healthy volunteers (Liu et al, 2017). The albumin-bound peptides usually called "*albuminomes*" have been classified into two distinct groups: (i) fragments of endogenous plasma proteins that are predominantly of high to medium abundance and (ii) peptides that are disease-related markers (Scumaci et al, 2011); the peptides produced from increased activity of cancers' proteases and some other unusual metabolisms mentioned above. And, the cancerous albuminomes may play an important role in albumin thermal stability.

Theory: Kinetically driven energetics for thermal denaturation of albumin

The thermal denaturation of albumin has been successfully described by a simple Lumry-Eyring model (Pico, 1997). Based on the model, thermal denaturation of a native protein of *N*-specie occurs in two steps; a) a reversible unfolding/refolding between *N* and unfolded *U*-specie, and b) an irreversible change from *U* to denatured *P*- specie:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \xrightarrow{k_3} P$$

Scheme I

Presumably, all of the rate kinetics (arrows labeled by the rate parameters in Scheme I) are first order having the temperature-dependent rate parameters k_1, k_2, and k_3 for reversible unfolding, refolding, and irreversible denaturation, respectively. And, chemical equilibrium between N and U is established and shows equilibrium parameters namely; $K = k_1/k_2$. Using these rate equations, Sanchez-Ruiz (1992) described the mole fraction of the native protein: x_N as being the exponential function of the absolute temperature, T:

$$x_N = \frac{1}{K+1}\exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T}\frac{k_3 K}{K+1}dT\right\} \tag{1}$$

Notice that the exponent is an integral form of an expression of temperature-dependent parameters from a low temperature T_0 where P-specie was negligible to a temperature T, and v is the rate of DSC temperature scanning.

There have been 2 possible cases: 1) the irreversible alteration (k_3 in Scheme I) is slowest and determined overall rate, i.e., $k_3 << k_2$ while K is far less than 1(Pico, 1997). As a result, N- and P-species are significantly populated. Thus, Scheme I can be approximately modified as:

$$N \xrightarrow{k_3 K} P \qquad \text{Scheme II}$$

Equation 1 becomes:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} k_3 \cdot K \cdot dT\right\} \tag{2}$$

And, 2) conditioned as $k_1 << k_3$ with $K << 1$, most of the albumin molecules of N-specie transit to the final state of P-specie and amount of U-specie is very low making the Scheme I to be:

$$N \xrightarrow{k_1} P \qquad \text{Scheme III}$$

Again, Equation 1 becomes:

$$x_N = \exp\left\{ -\frac{1}{\upsilon} \int_{T_0}^{T} k_1 \cdot dT \right\} \tag{3}$$

The bound ligands may affect the albumin denaturation which yields the approximation of the process flavor either Scheme II or III relatively depending on the rate kinetics of the steps in Lumry-Eyring model. In addition, Equations 2 and 3 can be generally written as:

$$x_N = \exp\left\{ -\frac{1}{\upsilon} \int_{T_0}^{T} k_{app} \cdot dT \right\} \tag{4}$$

where, k_{app} is an apparent first order rate parameter; either $k_{app} = Kk_3$ or $k_{app} = k_1$.

Furthermore, k_{app} can be described as an Arrhenius type exponential function:

$$k_{app} = \exp\left\{ -\frac{E_{app}}{R} [\frac{1}{T} - \frac{1}{T_k}] \right\} \tag{5}$$

where, E_{app} is the apparent activation energy, T_k is the temperature at which k is equal to 1 min^{-1} corresponding to the Arrhenius constant (Sanchez-Ruiz, 1992). Introducing Equation 5 to Equation 4 yields:

$$x_N = \exp\left\{ -\frac{1}{\upsilon} \int_{T_0}^{T} \exp[-\frac{E_{app}}{R}(\frac{1}{T} - \frac{1}{T_k})] \cdot dT \right\} \tag{6}$$

The integral form of the exponent in Equation 6 does not have an analytical solution facing difficulty in non-linear data fitting to obtain E_{app}. But fortunately, it can be simplified by imposing a rational approximation (Khrapunov and Brenowitz, 2011) as:

$$\int_{0}^{T} \exp(-\frac{E_{app}}{R \cdot T}) dT \approx \exp(-E_{app}/RT) \cdot \frac{T^2}{E_{app}/R} \tag{7}$$

which, in turn, converts Equation 6 to:

$$x_N = \exp\left\{ -\frac{1}{\upsilon} \exp(E_{app}/RT_k) \cdot \frac{T^2 \cdot \exp(-\frac{E_{app}}{RT})}{\frac{E_{app}}{R}} \right\} \tag{8}$$

Equation 8 would be valid with reasonably low errors only for $T < 370$ K (Khrapunov and Brenowitz, 2011).

By using Equation 8, Faroongsarng and Kongprasertkit (2014) and Faroongsarng (2016) were able to determine the energetic information for albumin thermally denatured with or without either fatty acid or drug ligands binding. It was found that the apparent activation energy (E_{app} in Equation 8) of free albumin thermal denaturation was significantly different from that of bound form. It was further suggested that ligand binding may cause denaturing kinetic energy level to transform from the level corresponded to the state dominated by irreversible change (Scheme II) to that by reversible unfolding (Scheme III) as illustrated in Figure 1.

In general, protein binding occurs in a reversible manner by means of weak interactions such as van der Waals force, hydrogen bonding, and hydrophobic interaction (Singh and Kishore, 2006). It is hypothesized that the cancerous albuminomes have significantly high binding affinities to albumin irrespective to their chemical structures. Thermal induced denaturation of either free or liganded albumin follows the above described Lumry-Eyring model. And, the modulation of albumin stability may occur via the cancerous albuminome binding interaction.

Objective

The study aimed at the investigation of blood plasma protein, emphasizing in albumin, stability modulation in breast cancer in various stages by means of DSC. It was to explore the potential of the machine for the disease diagnosis. In addition, normal plasma drawn from healthy volunteers was profiled to recognize the presence of albumin in DSC thermogram.

วิธีการทดลอง

Experimental

Blood plasma collection

50 female healthy volunteers were recruited from the persons who came to Songkla-nakarind hospital for blood donation. It is because their plasma thermograms may serve as the references for future diagnosis of CA breast. The protocol of blood collection has been approved by Ethics Committee, Faculty of Medicine, Prince of Songkla University. After routine screening, the subjects were asked to participate the project with their voluntary decision. After freely agreed, the subjects signed informed consent. In addition to routine blood donation, the volunteers were asked to give approximately 12 ml of blood sample into EDTA blood collection tubes. After collection, the blood was gently mixed by inverting the tube 8 to 10 times and stored at 4ºC until centrifugation. Within four hours of blood collection, blood samples were centrifuged in a horizontal rotor (swing-out head) for 10 to 20 minutes at 1,100-1,300 X g at room temperature. After centrifugation, plasma layer was carefully collected with an appropriate transfer pipette and stored at -80 degrees C before use.

Subject/Specimen inclusion criteria	Subject/Specimen exclusion criteria
☐ Female aged ≥ 18 years; ☐ Willing and able to provide consent; ☐ Performance status Eastern Cooperative Oncology Group (ECOG) 0-2; ☐ Newly diagnosed as breast cancer and naive to drug therapy; ☐ The stage of the cancer was determined according to the oncological criteria of macroscopic description and morphological analysis (American Joint Committee on Cancer; AJCC).	☐ Pregnant or lactation; ☐ diagnosed as other cancers; ☐ Evidence by physical examination or mammography of other suspicious masses, densities, or micro-calcifications in either breast, unless biopsied and found to be benign; ☐ Any prior treatment; ☐ Patients with severe co-extensive comorbidities or significant psychiatric illness

Table 1 Inclusion/Exclusion criteria for recruiting subjects to participate the study

Patients with breast cancer in various stages who visited the hospital and fulfilled criteria listed in Table 1 were recruited. It is noted that the tumor, node, and metastasis staging system developed by the American Joint Committee on Cancer (AJCC) in collaboration with the Union for International Cancer Control (UICC) referred to as the AJCC TNM staging system (Gress et al, 2017) was employed. The protocol of blood collection has been approved by Ethics Committee, Faculty of Medicine, Prince of Songkla University (Protocol No: 60-129-10-1, Approval date: July 5, 2017). After screening, the subjects were asked to participate with their voluntary decision. After freely agreed, the subjects signed informed consent. The volunteers were asked to give approximately 12 ml of blood sample. The blood samples were treated in the same manner of those from healthy volunteers described above.

Plasma samples and the preparation of albumin-enriched fraction

Each of the frozen plasma samples was thawed to room temperature. The liquid sample was centrifuged to separate endogenous lipids. The first portion of each sample was directly subjected to the DSC run whereas the second one was pretreated to obtain the albumin-enriched fraction out of the sample. The fraction was prepared from individual plasma samples using the method modified from Gundry et al (2007). Briefly, after thawing, the plasma was mixed with 42% ethanol/100 mM sodium chloride in equal volume and incubated at 4°C for 1 hour. The mixture was then centrifuged at 16,000 X g for 45 min. The clear supernatant which was the albumin-enriched fraction was collected and stored at 4°C prior to the DSC experiment.

The investigation of thermal denaturation of plasma proteins by mean of differential scanning calorimetry

The differential scanning calorimeter (DSC 8000, Perkin-Elmer Crop., Norwalk, CT, USA) previously calibrated with standard Zinc and Indium to ensure the accuracy and precision of the obtained heat of transitions and for the corresponding temperatures was employed. The machine equipped with a cooling accessory (Perkin-Elmer Intercooler II) and purged by nitrogen gas. ~15 μL of liquid sample was accurately weighed and placed in a tightly sealed aluminum pan. The individual sample pan was subjected to run against an empty pan as a reference. With a loading temperature of 25 °C, the sample was pre-heated from 25 °C to 37°C, equilibrated at 37°C for 15 min, and subsequently scanned from 37°C to 90°C at a rate of 5.0°C/min. Then, it was cooled down to 37°C. The sample was re-heated with identical conditions of the first run. Typically, the first DSC scan displays the thermal denaturation profile of the native plasma proteins, while the second scan displays thermogram without detectable thermal event. This second scan serves as the instant baseline for each of individual profiles. Duplication was performed for each of the samples. All the DSC thermograms were analyzed using Pyris® software (version 11.0.0.0449, Perkin-Elmer Perkin-Elmer Crop., Norwalk, CT, USA).

ผลการทดลองและบทวิจารณ์

Results and Discussion

Demographic data of subjects

Subject	Healthy	Breast cancer
n	50	11
Age; Year ($\bar{X}$±s.d.)	36.2 ± 10.1	49.7 ± 3.9
BW; kg ($\bar{X}$±s.d.)	60.9 ± 12.2	58.4 ± 9.8
Height; cm ($\bar{X}$±s.d.)	158.8 ± 5.6	151.2 ± 9.2

Table 2 The demographical portrait of female subjects who enrolled in the study.

Between October 2017 and April 2018, there were 50 healthy and 11 patient female subjects with identical ethnic (Thai) aged 18-57 and 44-55 years old, respectively, enrolled to the study. The basic demographic data are summarized in Table 2. It was found from the Table that the body mass indexes (BMI in mean ± s.d.) were 24.97±4.02 and 26.04±7.33 kg/m^2 for healthy and patient subjects, respectively. They were considered statistically non-significant different and slightly overweighed. Recently, the global BMI has shifted toward a higher value as the prevalence of overweight/obesity has increased by 5% every decade with more rapid in females than males (Arnold et al, 2016). In addition, excess body weight has been associated with an increased risk of non-communicable diseases while female obesity was linked with poor breast cancer survival (Chan et al. 2014). Among the patient volunteers, two, five, and four subjects were in early, middle, and last stages of ductal carcinoma, respectively. All had given birth to 1-4 children with breast feeding. And, 7 out of 11 were menopausal women. In term of demographics described above, it may be assumed that the subjects came from an identical population. Thus, it is reasonable to assume that the endogenous composition of blood plasmas disregarding with those derived from breast cancer may vary homogeneously within a population's range. This assumption allows one to compare the blood plasma thermograms between normal and disease states

DSC thermograms of plasma proteins from healthy volunteers

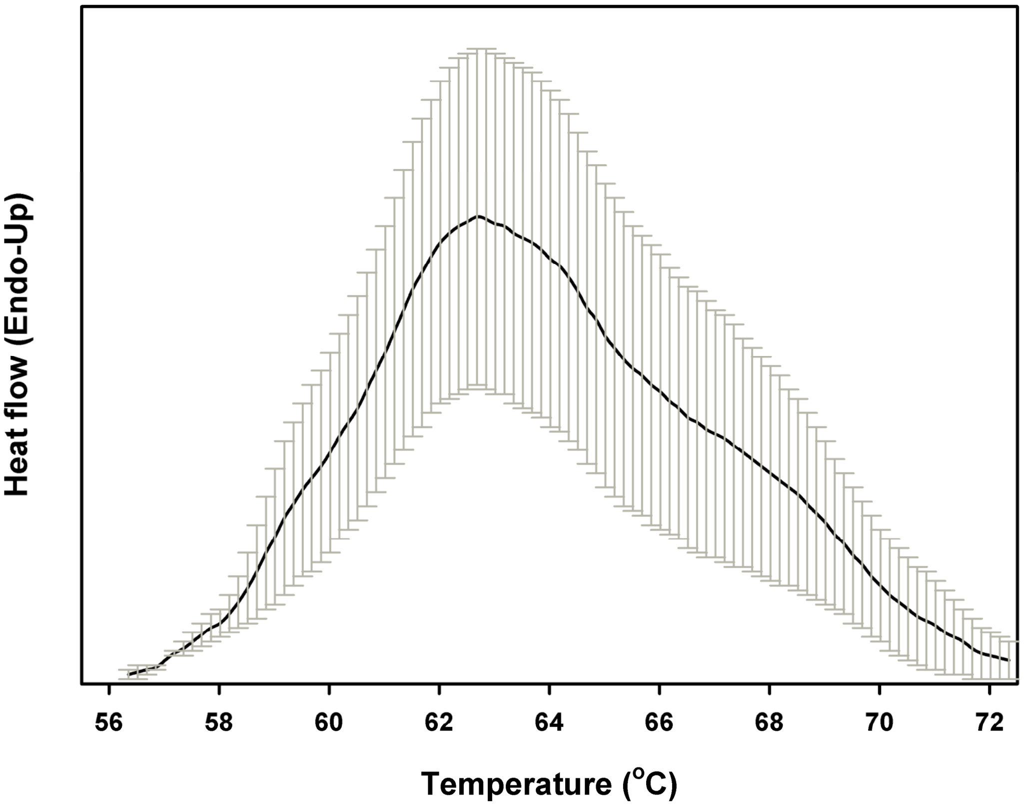

Figure 1 The average DSC thermogram of plasma samples under study. The data were taken from 50 female healthy volunteers. The standard error (error bar) for each of the coordinates demonstrates the significant inter-individual variations.

Figure 1 illustrates the average DSC thermograms of plasma proteins from healthy volunteers. As seen in Figure 1, the heat induced denaturation of plasma proteins is an endothermic event. It is noted also that this is an average profile from 50 individual volunteers showing relatively high inter-individual variations (error bars in Figure 1). Figure 1 was essentially similar to a number of normal plasma/serum thermograms previously reported (e.g. Koynova et al, 2018; Kędra-Królik et al, 2017; and Garbett at al., 2008). Human plasma is a complex fluid containing more than 3,000 individual proteins and peptides. Thus, its thermogram may be a weighed-combination of the proteins' thermal transition. As it is a weight-combination, only a few most abundant proteins present in plasma determine the thermogram. Garbett at al. (2008) demonstrated that the summation of weighed thermograms of the individual purified proteins including albumin, IgG, fibrinogen, transferrin, IgA, α_2-macroglobin, α_1-antitrypsin, complement C3, IgM, and haptoglobin accurately reproduced the features of average healthy plasma thermogram. So, the thermogram provides a unique signature of plasma proteins.

DSC thermogram of albumin-enriched fraction

It was suggested that interactions among abundant plasma proteins themselves were negligible (Garbett at al., 2008) allowing one to trace the convolving abundant proteins out of the endotherm of normal plasma. Koynova et al (2018) indicated 4 most abundant plasma proteins de-convoluted out of their thermogram of blood plasma from a healthy individual with relative amount ranked in descending order as albumin, globulins, IgG/transferrin, and fibrinogen. In addition to DSC endotherms of albumin-enriched fraction, the deconvolution of normal plasma endotherms into individually detectable protein transitions was done in this study. Figure 2 illustrates the deconvolution analysis of an endotherm of normal plasma (a) as well as that of albumin-enriched fraction (b). As seen in Figure 2 (a), the deconvolution revealed that there were at least thermal transitions of 4 abundant proteins that were consistent with those reported by Koynova et al (2018).

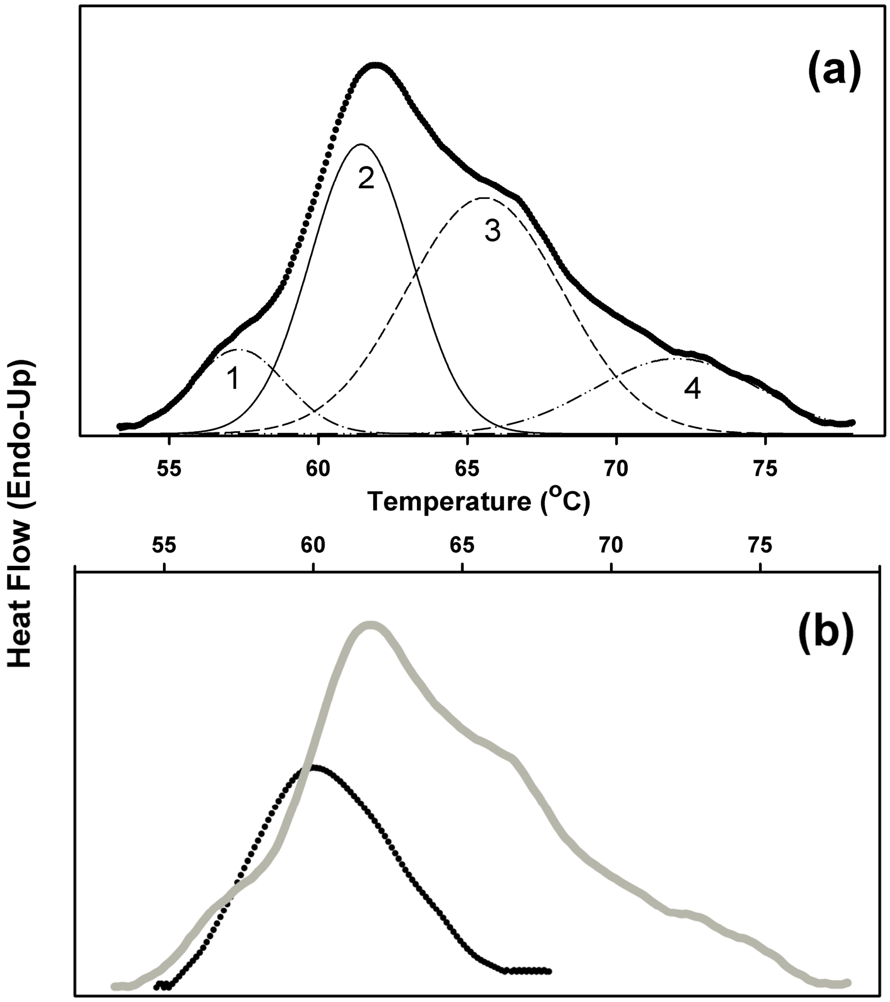

Figure 2 (a) The deconvolution of a DSC endotherm of individual plasma showing at least 4 detectable protein transitions. Notice that Gaussian curves were utilized in the analysis. (b) The DSC endotherm of the albumin enriched fraction compared with that of original plasma (gray profile).

Albumin constitutes approximately half of the plasma protein mass (Garbett et al, 2007); the highest among the proteins. Its thermal transition may be positioned at the apex of the thermogram (Garbett et al, 2008; Koynova et al, 2018) which corresponds to peak 2 in Figure 2 (a). Moreover, DSC tracing of enriched albumin fraction appeared close to peak 2 (Figure 2 (b)) but slightly shifted downward. It has been stated that a native protein with ligands always denatured at somewhat higher temperature regardless of the structural consequences (Sanchez-Ruiz, 2007). Several endogenous substances present in normal plasma may behave as binding ligands for albumin. These ligands may be eliminated during the process of albumin enrichment. As a result slight decrease in denaturing temperature was observed. However, the average temperature of transition of albumin enriched fraction was not significantly different from that of peak 2 (Table 2). Or in other words, these endogenous ligands might not be sufficient to modify albumin stability.

DSC Thermograms of plasma protein from breast cancer patients

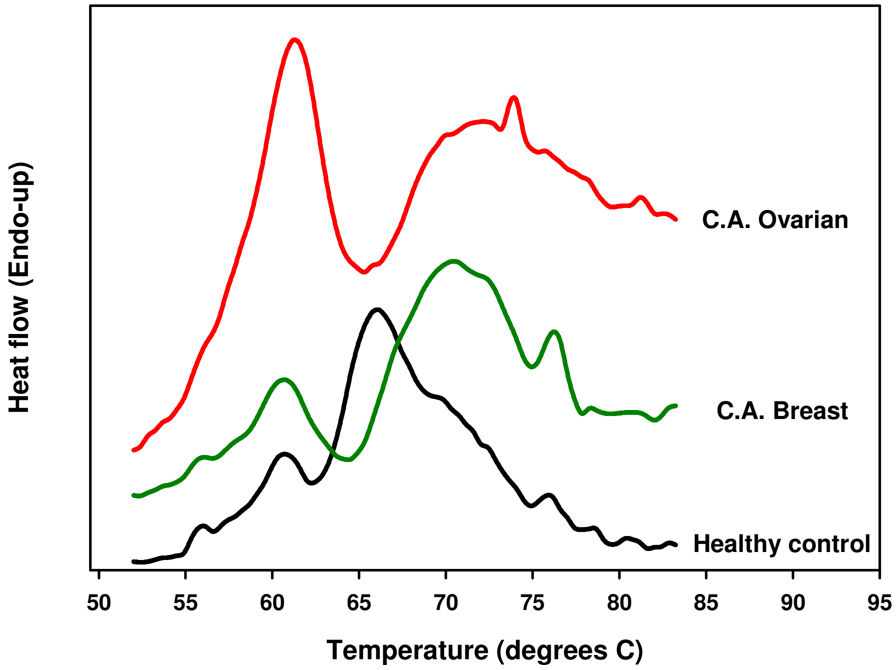

Figure 3 The DSC thermograms (thermal signatures) of plasma proteins compared among disease states

Figure 3 demonstrates the thermograms of plasma proteins from a healthy subject and individual patients (Breast and Ovarian cancers labeled in the Figure). As seen in Figure 3, the signature of thermal denaturation of plasma proteins obtained from a healthy volunteer markedly differed from those from disease volunteers. The deconvolution analyses into individually detectable protein transitions of each of the thermograms are showed in Figure 4.

As seen in Figure 4, DSC thermograms resulted in 4-6 apparently co-existing transitions and their relative compositions changed with the disease states. It is noted that this convolution was based on the individual plasma. It might somewhat differently exhibit the detectable transition compared to the average one as the small individual transitions might cancel out resulting in less apparent detectable transitions on average thermogram. The most abundance one which is albumin may be designated as Peak 2 (Figure 4 (a)). It is because it approximately appeared at the albumin denaturing temperature previously reported by Faroongsarng and Kongprasertkit (2014). It is also observed that albumin's peak transition of the disease states was increased (indicated as arrows in Figure 4), i.e., the disease states made albumin "more stabilized" to thermal agitation. As previously mentioned, albumin has a great binding capability. Thus, thermal stabilized non-ligand albumin can be explained by binding of cancer-specific makers to albumin (Kikalishvili et al, 2015).

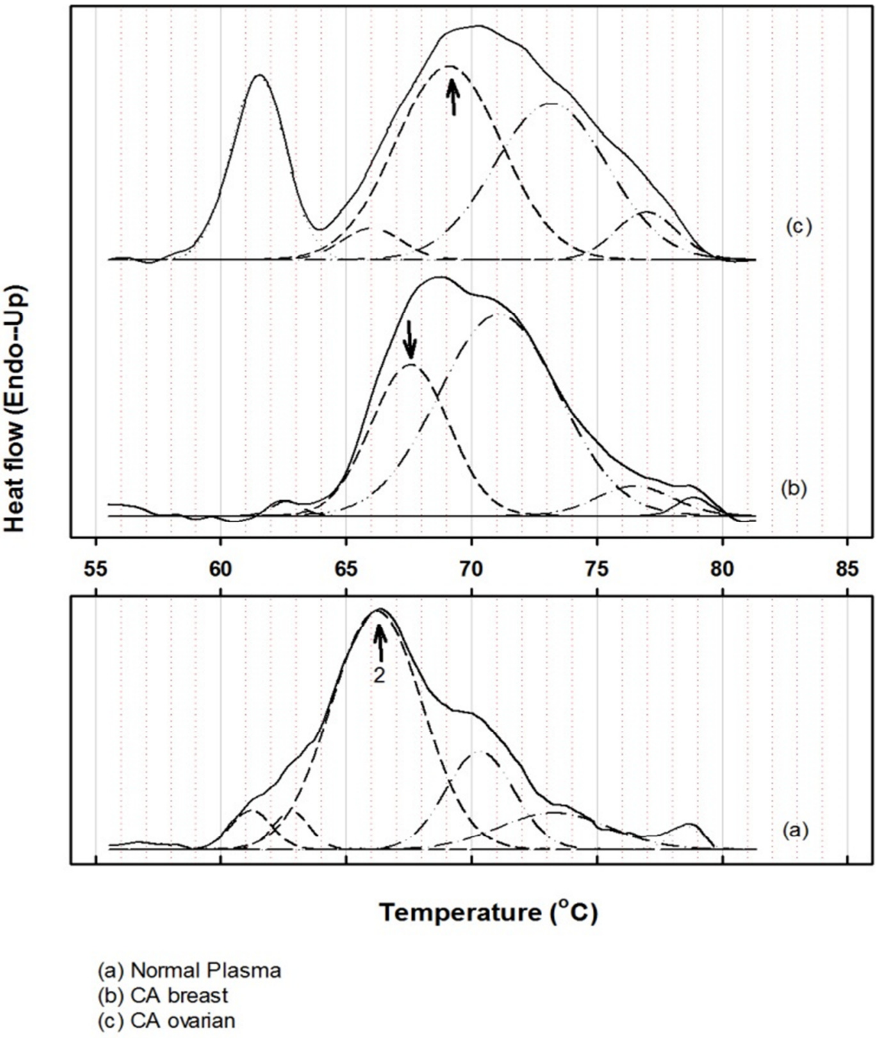

Figure 4 Deconvolution analyses of DSC thermograms of plasma from healthy subjects (a), a patient with CA breast (b), and that with CA ovarian (c) demonstrating about 6 dominating proteins presented in human plasma.

Peak 2: the plasma albumin’s thermal transition from breast cancer patients with various stages

Figure 5 displays the DSC thermograms of blood plasma from female individuals with different stages of breast cancer compared to that of normal one (a gray curve in Figure 5). For clarity, the corresponding Peaks 2 are also illustrated in the inset of Figure 5. It is seen that the denaturing temperature of plasma albumin increased with the progression of the disease. The temperature increasingly varied form 61.90 °C for breast cancer with early stage to 73.34 °C for that with last stage compared to 61.52±0.49 °C for healthy volunteers (n = 50). On the contrary, it looks that the albumin-enriched fractions prepared from patients’ plasma did not clearly reveal the trend of temperature increment. Again, albumin may loss its albuminomes during the process of enrichment. As a result, the increased pattern of denaturing temperature was not clearly observed.

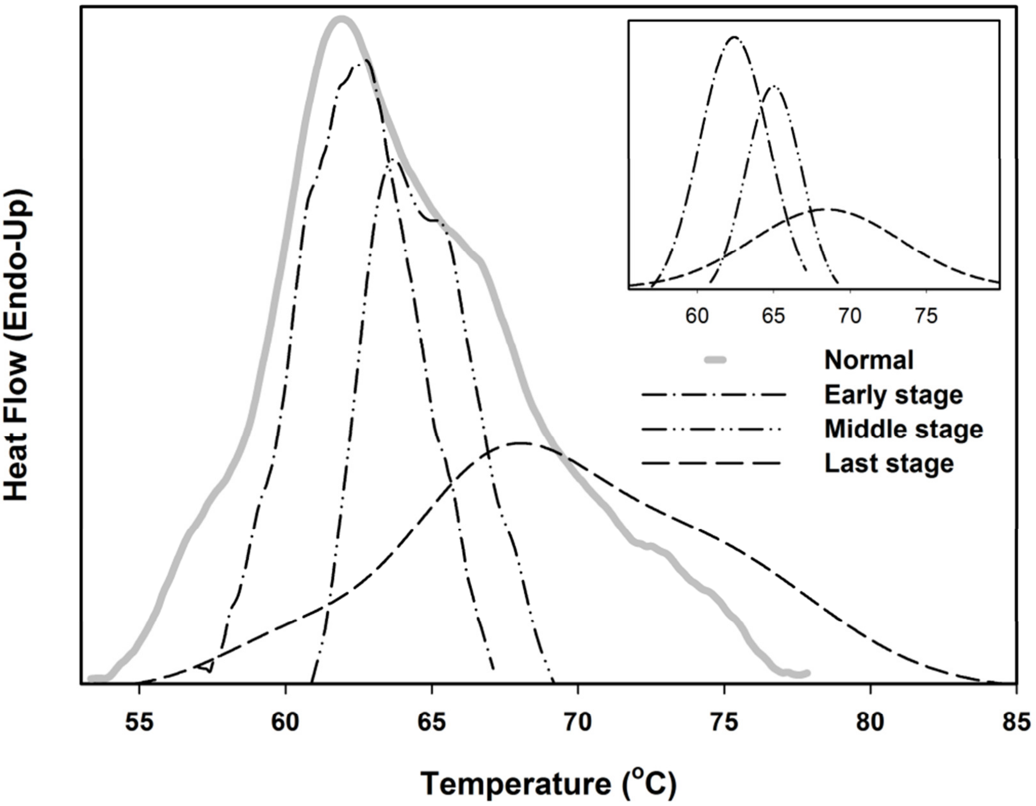

Figure 5 The DSC thermograms of blood plasma from the individuals with different stages of breast cancer. The gray curve is thermogram of that form healthy individual. The inset shows the corresponding de-convoluted peaks out of the figure’s thermograms (Peaks 2) which are expected to be albumin.

Denaturing temperature of plasma albumin *vs*. breast cancer staging

In peptide biomarker studies, proteomic/peptidomic analyses using advanced comprehensive separations coupled with mass spectrometry (e.g. matrix assisted laser desorption /ionization time-of-flight MS (Kim et al. 2016), and tandam Fourier transform MS (Shen et al, 2010) have been primarily done on blood plasma samples. They offer specific peptide identification as well as quantification. Nevertheless, no single peptide but the changes in pattern of a bunch of peptides emerges from the analyses as a whole reliable biomarker for such a disease (Shen et al, 2010). Those tumor peptides might turn to be albuminomes yielding the modification of stability of albumin as illustrated in Figure 5. Similar to these findings, albumin peak of DSC thermo-gram was previously found to shift to higher temperature as the ovarian cancer progressed (Kim et al, 2016). It was suggested that the increased temperature was due to the disease-generated albuminome binding interaction (Garbett et al, 2007) as significantly high peptide expression with upward-shifted thermograms was observed (Kim et al, 2016). It has been further evidence that the degree of cancerous invasion was associated with the extent to which proteins had been degraded. For breast cancer, 839 distinct peptides were profiled compared with 425 peptides for healthy controls. The ratios of tumor peptide relative abundance can increasingly vary up to more than 4,000 fold between breast cancer in late stages and normal plasma (Shen et al, 2010). Also, the degradation could provide the tumor albuminomes in significant amount to markedly increase transition temperature of albumin especially in the very late stage of cancerous invasion. On the other hand, other high abundant proteins such as IgG could also bind to these peptide ligands (Kim et al, 2016). But, IgG appeared as a shoulder, right after an apex of Peak 2 of the thermogram (Kim et al, 2016), and its relative amount varied considerably due to the complications of disease pathologies as well as patient conditions. Thus, IgG was not chosen so as not to face the difficulties of data analysis.

The physician's conventional tumor staging (e.g. stage-I, -II, -III, and -IV) indicates the cancerous invasion qualitatively. Breast cancer is heterogeneous. Its key prognostic factors include primary tumor size (T), number and size of invaded regional lymph nodes (N), and the absence or presence of metastasis (M). Furthermore, these factors possess notations usually presenting as a number (e.g. 0, 1, 2,…) to describe the degrees of tumor invasion, for examples, size or extent of the tumor, existence and extent of regional lymph node involvement, and whether distant metastasis is present. The higher the number, the more invasive it will be. It has been stated that the cancer staging was based on aggregate information resulting from T, N, and M category designations (Gress et al, 2017). Thus, summation of the numbers of categories may primarily quantitate the tumor stage. The so-called tumor staging score; the summation of the numbers of notations for the key prognostic factors was plotted against the denaturing temperature of albumin in blood plasma and illustrated in Figure 6. It turned out that the denaturing temperature was linearly proportional to the staging score with a good statistical correlation (r^2 of 0.9666: presented as a dash line in Figure 6). Presumably, the increase in denaturing temperature was directly dependent on amount of albuminomes. Thus, the increased denaturing temperature may then imply the increase in albuminome abundance produced by the tumor invasion as breast cancer progressed.

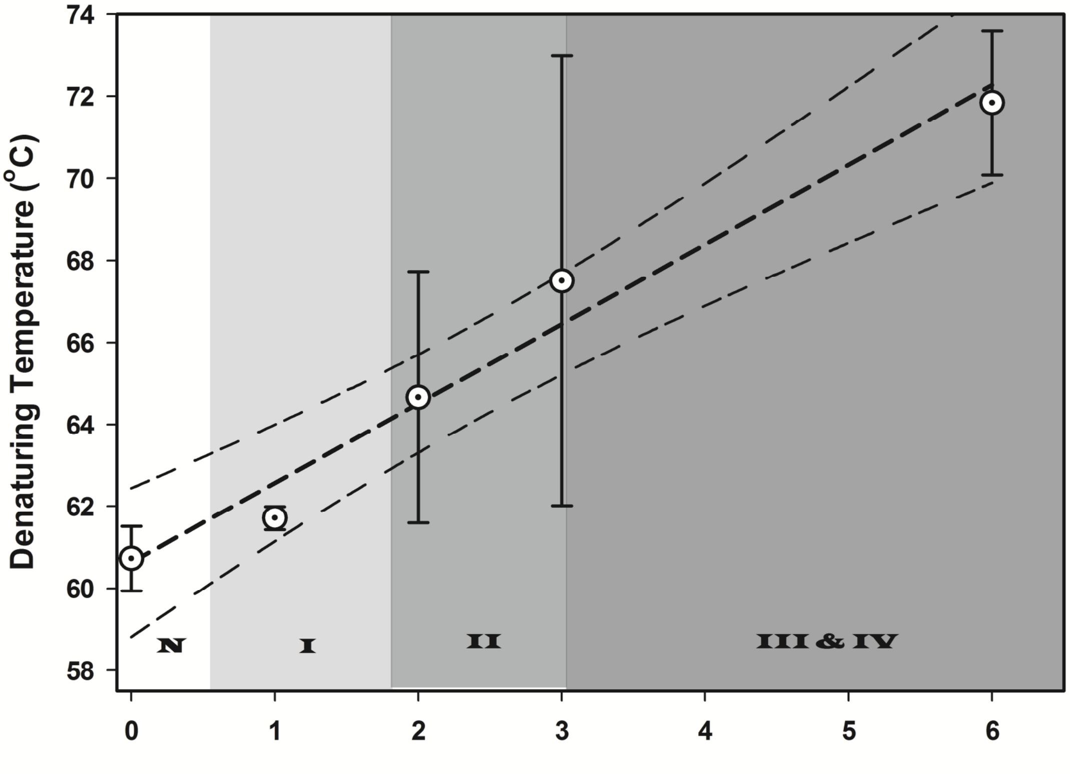

Figure 6 The plot between primary tumor staging score and denaturing temperature of albumin of breast cancer volunteers. The shade areas indicate the staging categories (I, II, and III&IV): the more invasions, the darker is. While, the area; N is for normal blood plasma. The dash lines represent a linear trend and its 95% confident interval.

Apparent activation energy for thermal denaturation of native albumin: the Data from Healthy volunteers

As described in theory section previously, non-linear fitting of Equation 6 yields the apparent activation energy exhibiting the kinetic state of thermal denaturation of an of interest protein. In this study, the thermogram of albumin-enriched fraction and that de-convoluted from normal plasma (peak 2) were on focused. Using the technique developed by Faroongsarng and Kongprasertkit (2014), x_N of Equation 6 can be estimated from the energetic information of the thermogram as: $x_N = 1 - F_T$, where F_T is the ratio between the amount of heat at absolute temperature; T and the total heat of transition of the entire DSC peak, i.e., the partial transition at corresponding T. It was found that all the regressions were successful with r^2-values higher than 0.98. Figure 7 illustrates an example of non-linear regression of x_N and T out of peak 2 from one of subjects. Notice that those out of albumin-enriched fraction exhibited the same pattern as Figure 7.

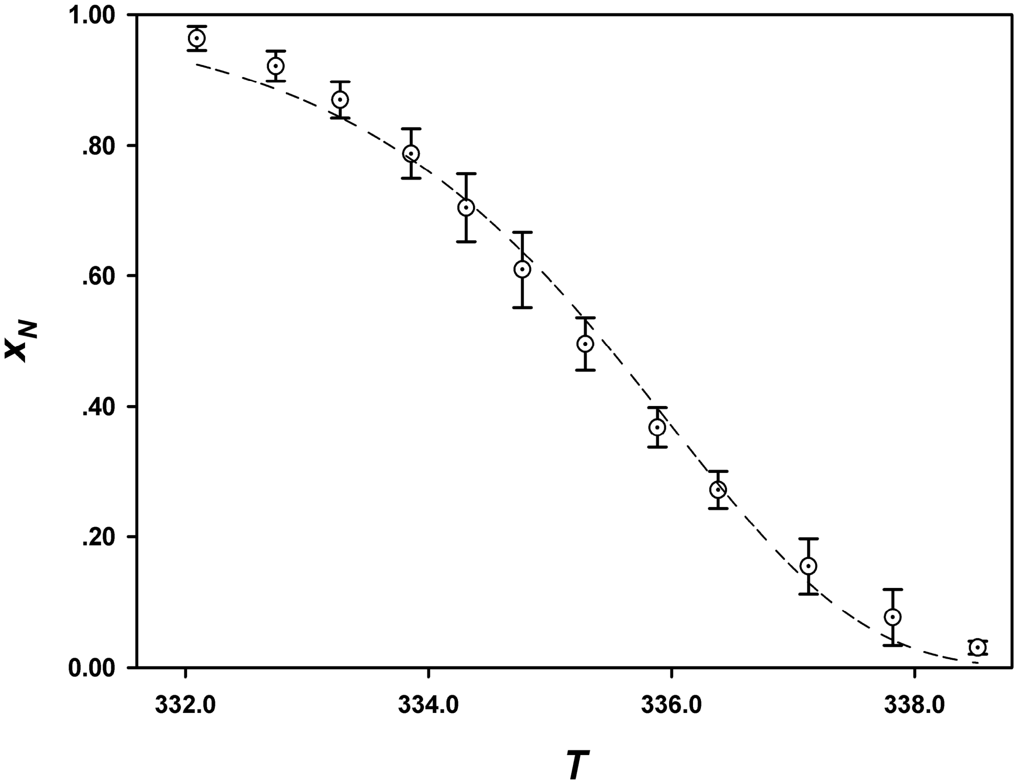

Figure 7 The plot between the estimated mole fraction of native albumin ($x_N = 1 - F_T$) and absolute temperature (T) showing a successful non-linear regression according to Equation 6 (broken line).

Table 3 summarizes the apparent activation energies and their denaturing temperatures of Peak 2 of thermograms from healthy volunteers as well as their enriched-albumin fractions. It is seen from Table 3 that E_{app} -values of denaturing albumin-enriched fraction were slightly lower compared to those of deconvolution counterparts whereas more deviation was observed. It may be because manually handling in the pretreatment protocol for albumin fractionation might introduce additional variations. Furthermore, adding 42% ethanol/100 mM sodium chloride in equal volume to a plasma sample could markedly reduce the DSC signals. As a result the data of enriched-albumin fraction exhibited considerably more variability than those of peak 2. These variations leaded one not to reject null hypothesis, i.e., the E_{app} -value of denaturing albumin-enriched fraction was not statistically significant from that of peak 2. Furthermore, the E_{app} –values were principally identical to that of commercial human serum albumin after the removal of stabilizers previously reported (E_{app} –value of 151.3 Kcal/mole: Faroongsarng and Kongprasertkit, 2014). Thus, the E_{app} –value from Peak 2-deconvolution could be valid for albumin energetic evaluation under thermal agitation.

Albumin	**E_{app}; Kcal/mole (mean $\pm$ s.d.)**	**^{3}Temperature; ^{o}C. (mean $\pm$ s.d.)**
^{1}Enriched fraction	127.79 $\pm$ 45.15	59.92 $\pm$ 1.49
^{2}Peak 2	141.08 $\pm$ 18.67	61.52 $\pm$ 0.49

Table 3 Apparent activation energies as well as denaturing temperatures obtained from non-linear data fitting according to Equation 6.

1. Based on the energetic information of DSC endotherm of albumin-enriched fraction from blood plasma of 50 individual female subjects.
2. Based on the energetic information of peak 2 in de-convoluted DSC endotherm of blood plasma of 50 individual female subjects.
3. Denaturing temperature was defined as a maximum temperature of an endotherm.

Energetic profile of thermal induced denaturation of plasma albumin with breast cancer: A potential indicator for breast cancer diagnosis

To have an energetic profile, not only E_{app}-value derived from the thermogram, but the concentration of ligand is needed. Quantification of peptide ligands produced by breast cancer of an individual subject was rather difficult as it required laborious and time consuming proteomic methods of determination to do so. Fortunately, it was not necessary to quantify the peptide albuminomes as the linear relationship showed in Figure 6 may infer the use of temperature as a representative of the amount of the tumor albuminome ligands. E_{app}-temperature "*energetic*" profile of thermal denaturation of albumin from blood plasma thermograms of breast cancer in various stages was constructed and showed in Figure 8. The sigmoidal ligand binding response model illustrated in Equation 9 was utilized as a model for statistical data fitting.

$$E_{app} = E_{min} + \frac{E_{max} - E_{min}}{1 + 10^{\log T_{0.5} \cdot \alpha}} \qquad (9)$$

In equation 9, E_{max} and E_{min} are E_{app} at the extreme ends, $T_{0.5}$ and α are transition temperature when E_{app} is halfway between extreme ends and hill coefficient which describes the steepness of the fitted curve, respectively. The data were very well fitted into the model having r^2 of 0.9265. It is seen that the energetic curve in Figure 8 apparently changed from one extreme to the other with temperature increment. And, it was found that the estimates (value ± standard error of estimation) of E_{app} at the extreme ends were 132.68±14.21 (E_{max}) and 46.76±8.41 (E_{min}) Kcal/mole for normal (denoted as N in Figure 6) and breast cancer with very last stage (denoted as III&IV) thermograms, respectively. In addition, the estimates of $T_{0.5}$ and α were 65.56±1.16 °C and -0.3491±0.1455, respectively. In case of albumin without ligand binding, it has been assumed based on Scheme II that the step of irreversible change was negligible for time scale at which the DSC was carried out (e.g. more than 1 °C/min scanning rate: 16), the heat associated with the protein denaturation may approach the enthalpy of unfolding (ΔH_U). It was previously reported that ΔH_U of un-liganded human albumin was in the range of 88.6-115 Kcal/mole depending on the experiment settings (Pico, 1997; Celej et al, 2006). The energetic state of blood plasma albumin of normal healthy subjects found in the current study was comparable to ΔH_U previously reported confirming that the denaturation could follow Scheme II. The presence of slight discrepancy might be due to either the differences of experimental settings or the contribution of energetics from some endogenous ligand binding (Faroongsarng and Kongprasertkit, 2014).

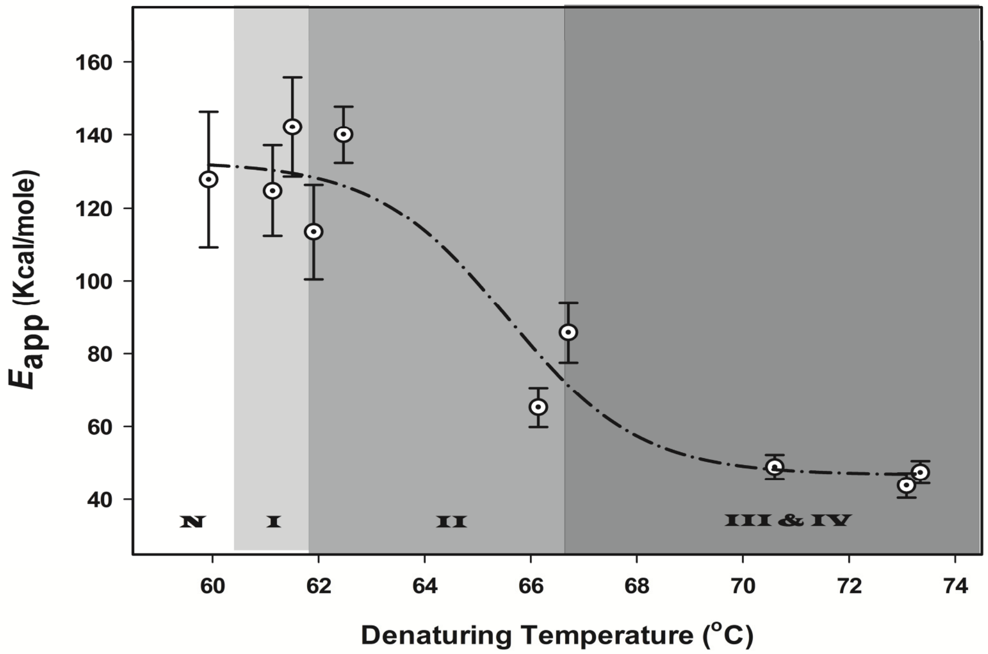

Figure 8 Energetic profile of albumin derived from DSC thermograms of blood plasma of breast cancer with various stages. Except that of normal plasma located in N-area which is the mean value with standard deviation of 50 data sets, the coordinates are the estimated values according to Equation 8 with their standard errors of estimation presented as vertical bars. A trend line is a non-linear fitted curve base on the model of sigmoidal ligand binding response. It shows how E_{app} transit from one state to other. The shade areas indicate the staging categories (I, II, and III&IV): the more invasions, the darker is. While, the area; N is for normal blood plasma.

During thermal induction, the protein may liberate ligands instantaneously before unfolding (Arakawa and Kita, 2000). Thus, increased amount of tumor peptides would shift the equilibrium towards albumin- albuminome complexes yielding the increase in temperature necessitated to unfold the protein. But, the phenomenon took place regardless of the structural consequences with contribution in only slight increased temperature (Sanchez-Ruiz, 2007). It might not be the case as the denaturing temperature of albumin with the late stages cancer was far higher compared with normal one, e.g. more than 10°C increased. Upon binding, it seems that the denaturing process attained different energetic state since the decreased E_{app} leveled off at a certain point (Figure 8). In the secondary structure, human albumin folds in a heart-shaped globule containing 3 helical domains indicated as domain-I, -II, and -III, each consists of sub-domains A and B. It was evident that un-liganded albumin showed remarkable difference in conformation compared with bound form (Ascenzi and Fasano, 2010). Among the albumin domains, sub-domain IIIA is prone to reversibly unfold due to relatively fewer interactions with other domains of the albumin molecule (Sugio et al, 1999). Moreover, domain III possesses high-affinity sites (Fanali et al, 2012) where the tumor albuminomes likely get associated with. It is possible that bound albuminomes might alter the albumin-albuminome complex conformation. This may cause the rate of reversible unfolding to relatively lower and become a rate determining step driving Scheme II kinetically turn to Scheme III as indicated by 2 different energetic states seen in Figure 8. E_{app} of Figure 8 may be the total activation energy of the weighed combination between the two depending on the presence of tumor albuminomes. Therefore, the energetic level in Figure 8 may be used as a diagnostic parameter that might indicate the progression of breast cancer.

As seen in Figure 8, the E_{app}-value only slightly decreased in early stage of cancerous invasion. It began considerably decreased in middle stage, and then flattened in last stage. To be a good disease-related indicator, the change of E_{app}-value should be observable within an early period of tumor invasion. It is because the difficulties in therapy for cancer with very late stages have been the main reason for high mortality rate. It would have been significantly reduced with prolonged life span if the disease could be diagnosed earlier. However, the magnitude of α-coefficient from data fitting was less than unity ($\alpha = -0.3491$) showing a shallow slope of the energetic curve that made the observable energetic reduction occur in the middle stage of breast cancer instead of the early one. It may be because the amount of cancerous albuminomes produced in early stage was not sufficient to make significant change in the energetic state during thermally induced denaturation. The determination of E_{app}-value out of blood plasma thermogram might be appropriate for the disease screening rather than early diagnosis. It was previously suggested that MS-based proteomic/peptidomic techniques should be done in addition to DSC. While DSC provided a signature thermogram indicative of a disease state, MS gave the specific details of cancerous albuminomes. They were mutually complementary in the diagnosis (Kim et al, 2016). A number of earlier works claimed DSC method as a potential tool for tumor diagnosis and monitoring (e.g. Vega et al, 2015; Zapf et al, 2011; Kim et al, 2016). But again, they did not concretely demonstrate the potential of the machine for early diagnosis. The pitfall of DSC to early detection of the disease is still challenging.

หนังสืออ้างอิง

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Faroongsarng, D., Sunpaweravong, S., Raksawong, A. 2019. Thermally Induced Denaturing Energetics of Human Blood Plasma Albumin by Differential Scanning Calorimetry (DSC) as an Indicator for Breast Cancer Diagnosis in Female Patients. *AAPS PharmSciTech* 20(4), 146. **Impact factor: 2.827 (2018)**

ภาคผนวก

(ประกอบด้วย reprint หรือ manuscript และบทความสำหรับการเผยแพร่)

AAPS PharmSciTech (2019) 20: 146
DOI: 10.1208/s12249-019-1356-5

Research Article

Thermally Induced Denaturing Energetics of Human Blood Plasma Albumin by Differential Scanning Calorimetry (DSC) as an Indicator for Breast Cancer Diagnosis in Female Patients

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Received 20 December 2018; accepted 23 February 2019; published online 18 March 2019

Abstract. Cancerous invasion yields unusual metabolisms providing a significant amount of peptide albuminomes that modulate albumin stability *via* binding. The study aimed at the investigation of the thermal stability of human plasma albumin with breast cancer of various stages by means of differential scanning calorimetry (DSC). Blood plasma was drawn from 11 female breast cancer patients and 50 healthy volunteers of homogeneous demographics. Plasma samples and their albumin-enriched fractions were subjected to DSC scanning between 37 and 90°C at 5°C/min rate. For normal blood plasma, a characteristic signature of DSC tracing was observed. And, the deconvolution of DSC thermograms revealed the recognition of thermal transition of albumin. It was found that denaturing temperature of albumin increased with increasing breast cancer staging which implied the increase in albuminome/peptide abundance produced by cancerous invasion. The analysis of albumin denaturing energetics based on rational approximation of the simple Lumry-Eyring model demonstrated that thermal transition of free albumin and albuminome-bound form attained energetic levels expressed as apparent activation energy ($E_{\mathrm{app}} \pm$ s.e.) of 132.68 ± 14.21 and 46.76 ± 8.42 Kcal/mol corresponded to the schemes dominated by irreversible alteration and by reversible unfolding, respectively. Thus, E_{app} value may indicate the degrees of cancerous invasion. It was proposed that E_{app} may be used as an indicator to diagnose and assess the prognosis of breast cancer.

KEY WORDS: differential scanning calorimetry; breast cancer; Lumry-Eyring model; human albumin; albuminome.

INTRODUCTION

Breast cancer has been ranked the number one female cancerous invasion in Thailand (1). To date, no single tumor marker for diagnosis was recognized. It is because the tumor invasion always involves many pathways of unusual protein/peptide metabolism. And, the marker targets in blood reflective of cancer at an early stage, if present, are in extremely low levels (2). Cancerous progression, invasion, and metastasis always associate with protein degradation catalyzed by cancer degradome proteases (3). The increased enzyme activities cause various low molecular weight protein substrate degradation peptides circulating in blood in a significant total amount. And, these cancerous peptides afford a potentially rich pool for the disease-related biomarker detection (4).

Differential scanning calorimetry (DSC) has been introduced to the field of proteomic study (5,6). The method offers physical basis of thermal stability of proteins because it monitors the heat changes associated with the thermally induced denaturation. DSC analysis of normal blood plasma yields a unique thermogram which is a thermal signature of denaturing transition of plasma proteins (6). Even though human blood plasma contains more than 3000 proteins and peptides, only a few of them, including albumin, IgG, fibrinogen, and transferrin, make up the majority of the mass. These few most abundant proteins determine the DSC thermogram as Garbett et al. (7) demonstrated that the summation of weighed thermograms of the individual purified proteins accurately reproduced the features of average healthy plasma one. It has been further found that the characteristic thermogram was altered due to the disease state (8–10). It is because pathologies and disorders trigger the change of plasma proteins composition which distorted thermogram, and the abnormality due to disease could modulate thermal stability of proteins (8). Thus, monitoring thermogram of blood plasma may lead to the disease diagnosis. A number of investigators reported that the changes of DSC calorimetric parameters namely denaturing

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transition temperatures, heat capacities, and enthalpies derived from blood plasma thermograms may aid the diagnosis of several diseases such as cervical cancer (6), colorectal cancer (10), and breast cancer (9–11). Unfortunately, the changes of plasma proteins composition detectable by DSC always occur as a consequence of common pathologies such as inflammations. While, the diagnosis requires the recognition of disease-related peptide "*biomarkers*", if present, which presents at such low levels that DSC cannot detect. But, monitoring the thermal stability modulation of high abundant plasma proteins may be possible as it was observed that the DSC peak of abundant proteins in blood plasma of breast cancer patients deviated to a higher temperature due to the disease state (11).

Among plasma proteins, albumin plays a major binding partner for ligands. It has been apparent that several small-molecule peptides, regardless of their chemical structures, bind with albumin (12). For instance, 141 peptides associated with albumin were found in a commercial albumin product (13) whereas 125 albumin-interacting proteins and 14 highly connected clusters were identified in blood plasma of healthy volunteers (14). The albumin-bound peptides usually called "*albuminomes*" have been classified into two distinct groups: (i) fragments of endogenous plasma proteins that are predominantly of high to medium abundance and (ii) peptides that are disease-related markers (15); the peptides are produced from increased activity of cancer degradome proteases and some other unusual metabolisms mentioned above. And, the cancerous albuminomes may play an important role in albumin thermal stability. The current study aimed at the investigation of blood plasma albumin stability modulation in breast cancer of various stages by means of DSC. It was to explore the potential of the machine for the disease diagnosis. In addition, normal plasma drawn from healthy volunteers was profiled to recognize the presence of albumin in DSC thermogram.

Kinetically Driven Energetics for Thermal Denaturation of Albumin

The thermal denaturation of albumin has been successfully described by a simple Lumry-Eyring model (16). Based on the model, thermal denaturation of a native protein of N-species occurs in two steps: (a) a reversible unfolding/refolding between N and unfolded U-species and (b) an irreversible change from U to denatured P-species.

Presumably, all of the rate kinetics (arrows labeled by the rate parameters in Scheme 1) are first order having the temperature-dependent rate parameters k_1, k_2, and k_3 for reversible unfolding, refolding, and irreversible denaturation, respectively. And, chemical equilibrium between N and U is established and shows equilibrium parameters, namely $K = k_1/k_2$. Using these rate equations, Sanchez-Ruiz (17) described the mole fraction of the native protein x_N as being the exponential function of the absolute temperature, T.

$$x_N = \frac{1}{K+1}\exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T}\frac{k_3K}{K+1}dT\right\} \tag{1}$$

Notice that the exponent is an integral form of an expression of temperature-dependent parameters from a low temperature T_0 where P-species was negligible to a temperature T, and v is the rate of DSC temperature scanning.

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} P$$

Scheme 1. The two-step denaturing transition based on a simple Lumry-Eyring model

There have been two possible cases:

1) The irreversible alteration (k_3 in Scheme 1) is slowest and determined the overall rate, *i.e.*, $k_3 << k_2$ while K is far less than 1 (16). As a result, N- and P-species are significantly populated. Thus, Scheme 1 can be approximately modified as:

Equation 1 becomes:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} k_3 \cdot K \cdot dT\right\} \tag{2}$$

2) Conditioned as $k_1 << k_3$ with $K << 1$, most of the albumin molecules of N-species transit to the final state of P-species and amount of U-species is very low making the Scheme 1 to be:

Again, Eq. 1 becomes:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} k_1 \cdot dT\right\} \tag{3}$$

The bound ligands may affect the albumin denaturation which yields the approximation of the processed flavor either Scheme 2 or 3 relatively depending on the rate kinetics of the steps in Lumry-Eyring model. In addition, Eqs. 2 and 3 can be generally written as:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} k_{app} \cdot dT\right\} \tag{4}$$

where, k_{app} is an apparent first-order rate parameter, either $k_{\mathrm{app}} = Kk_3$or $k_{\mathrm{app}} = k_1$. Furthermore, k_{app} can be described as an Arrhenius type exponential function:

$$k_{\mathrm{app}} = \exp\left\{-\frac{E_{\mathrm{app}}}{R}\left[\frac{1}{T}-\frac{1}{T_k}\right]\right\} \tag{5}$$

where E_{app} is the apparent activation energy, T_k is the temperature at which k is equal to 1 min^{-1} corresponding to the Arrhenius constant (17). Introducing Eq. 5 to Eq. 4 yields:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T}\exp\left[-\frac{E_{app}}{R}\left(\frac{1}{T}-\frac{1}{T_k}\right)\right] \times dT\right\} \tag{6}$$

The integral form of the exponent in Eq. 6 does not have an analytical solution facing difficulty in non-linear data fitting

$$N \xrightarrow{k_3K} P$$

Scheme 2. The modification of Scheme 1 where the denaturing transition is dominated by irreversible alteration

$$N \xrightarrow{k_1} P$$

Scheme 3. The modification of Scheme 1 where the denaturing transition is dominated by reversible unfolding

to obtain E_{app}. But fortunately, it can be simplified by imposing a rational approximation (18) as:

$$\int_0^T \exp\left(-\frac{E_{\mathrm{app}}}{R\cdot T}\right)dT \approx \exp\left(-{}^{E_{\mathrm{app}}}/_{RT}\right)\times\frac{T^2}{E_{\mathrm{app}}/R} \qquad (7)$$

which, in turn, converts Eq. 6 to:

$$x_N = \exp\left\{-\frac{1}{\upsilon}\exp\left({}^{E_{\mathrm{app}}}/_{RT_k}\right)\cdot\frac{T^2\cdot\exp\left(-\frac{E_{\mathrm{app}}}{RT}\right)}{\frac{E_{\mathrm{app}}}{R}}\right\} \qquad (8)$$

Equation 8 would be valid with reasonably low errors only for $T < 370$ K (18).

By using Eq. 8, Faroongsarng and Kongprasertkit (19) and Faroongsarng (20) were able to determine the energetic information for albumin thermally denatured with or without either fatty acid or drug ligands binding. It was found that the apparent activation energy (E_{app} in Eq. 8) of free albumin thermal denaturation was significantly different from that of bound form. It was further suggested that ligand binding may cause denaturing kinetic energy level to transform from the level corresponded to the state dominated by irreversible change (Scheme 2) to that by reversible unfolding (Scheme 3) as illustrated in Fig. 1.

In general, protein binding occurs in a reversible manner by means of weak interactions such as van der Waals force, hydrogen bonding, and hydrophobic interaction (21). It is hypothesized that the cancerous albuminomes have significantly high binding affinities to albumin irrespective to their chemical structures. Thermal-induced denaturation of either free or liganded albumin follows the above described Lumry-Eyring model. And, the modulation of albumin stability may occur *via* the cancerous albuminome binding interaction.

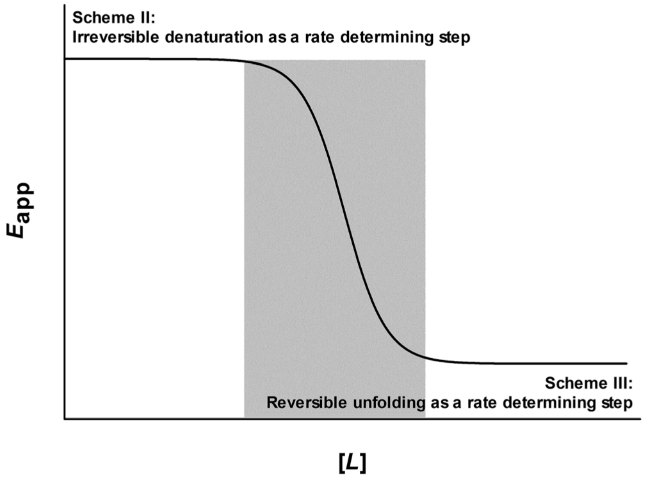

Fig. 1. Energetic profile showing the change of energetic state of denaturing kinetics of albumin as a function of ligand binding interaction proposed by Faroongsarng and Kongprasertkit (19). E_{app} and [L] are apparent activation energy obtained from data fitting according to Eq. 8 and ligand concentration, respectively. Shade area represents the area of transition from Schemes 2 to 3

MATERIALS AND METHODS

Blood Plasma Collection

Thai female healthy volunteers who came to Songklanakarind hospital for blood donation and patients with breast cancer of various stages who visited the hospital and fulfilled criteria listed in Table I were recruited. It is noted that the tumor, node, and metastasis staging system developed by the American Joint Committee on Cancer (AJCC) in collaboration with the Union for International Cancer Control (UICC) referred to as the AJCC TNM staging system (22) was employed. The protocol of blood collection has been approved by Ethics Committee, Faculty of Medicine, Prince of Songkla University (Protocol No: 60-129-10-1, approval date: July 5, 2017). After screening, the subjects were asked to participate in their voluntary decision. After freely agreed, the subjects signed informed consent. The volunteers were asked to give approximately 12 ml of blood sample into EDTA blood collection tubes. After collection, the blood was gently mixed by inverting the tube 8 to 10 times and stored at 4°C until centrifugation. Within 4 h of collection, blood samples were centrifuged in a horizontal rotor (swing-out head) for 15 min at 1100–1300×*g* at room temperature. After centrifugation, plasma layer was carefully collected with a transfer pipette and stored at − 80°C before use.

Plasma Samples and the Preparation of Albumin-Enriched Fraction

Each of the frozen plasma samples was thawed to room temperature. The liquid sample was centrifuged to separate endogenous lipids. The first portion of each sample was directly subjected to the DSC run whereas the second one was treated to obtain the albumin-enriched fraction out of the sample. The albumin-enriched fraction was prepared from individual plasma samples using the method modified from Gundry et al. (23). Briefly, after thawing, the plasma was mixed with 42% ethanol/100 mM sodium chloride in equal volume and incubated at 4°C for 1 h. The mixture was then centrifuged at 16,000×*g* for 45 min. The clear supernatant which was the albumin-enriched fraction was collected and stored at 4°C prior to the DSC experiment.

The Investigation of Thermal Denaturation of Plasma and Albumin-Enriched Fraction by Means of Differential Scanning Calorimetry

The differential scanning calorimeter (DSC 8000, Perkin-Elmer Crop., Norwalk, CT, USA) previously calibrated with standard zinc and indium to ensure the accuracy and precision of the obtained heat of transitions and the corresponding temperatures was employed. The machine equipped with a cooling accessory (Intercooler II, Perkin-Elmer Crop., Norwalk, CT, USA) and purged by nitrogen gas. ~ 15 μL of liquid sample, either plasma or albumin-enriched fraction, was accurately

Table I. Inclusion/Exclusion Criteria for Recruiting Subjects to Participate the Study

Subject/specimen inclusion criteria	Subject/specimen exclusion criteria
• Female aged ≥ 18 years • Willing and able to provide consent • Performance status Eastern Cooperative Oncology Group (ECOG) 0–2 • Newly diagnosed as breast cancer and naive to drug therapy • The stage of the cancer was determined according to the oncological criteria of macroscopic description and morphological analysis (American Joint Committee on Cancer (AJCC))	• Pregnant or lactating • Diagnosed as other cancers • Evidence by physical examination or mammography of other suspicious masses, densities, or micro-calcifications in either breast, unless biopsied and found to be benign • Any prior treatment • Patients with severe co-extensive comorbidities or significant psychiatric illness

weighed and placed in a tightly sealed aluminum pan. The individual sample pan was subjected to run against an empty pan as a reference. With a loading temperature of 25°C, the sample was pre-heated from 25to 37°C, equilibrated at 37°C for 15 min, and subsequently scanned from 37to 90°C at a rate of 5 .0°C/min. Then, it was cooled down to 37°C. The sample was re-heated with identical conditions of the first run. Typically, the first DSC scan shows the thermal transition profile of the plasma proteins, while the second scan displays thermogram without detectable thermal event. This second scan serves as an instant baseline for each of individual profiles. Duplication was performed for each of the samples. All the DSC thermograms were analyzed using Pyris® software (version 11.0.0.0449, Perkin-Elmer Perkin-Elmer Crop., Norwalk, CT, USA).

Equation 8 gives the apparent activation energy (E_{app}) exhibiting the kinetic state of thermal denaturation of plasma albumin. Using the technique developed by Faroongsarng and Kongprasertkit (19), x_{N} of Eq. 8 can be estimated from the energetic information of a DSC thermogram as $x_{\mathrm{N}} = 1 - F_T$, where F_T is the ratio between the amount of heat at absolute temperature, T, and the total heat of transition of the entire peak. The non-linear regression between x_{N} and T based on Eq. 8 was done by commercial software (OriginPro 2017, Origin Lab Corporation). To have the energetic information of albumin in blood plasma thermogram, a DSC tracing was de-convoluted based on Gaussian function and a peak with designated as blood plasma albumin was subjected to the above analysis. And, the energetic information of albumin-enriched fraction was obtained from the whole thermogram. It was found that all of the regressions were successful with r^2 values higher than 0.98.

RESULTS AND DISCUSSION

Demographic Data of Subjects

Between October 2017 and April 2018, there were 50 healthy and 11 patient female subjects with identical ethnic (Thai) aged 18–57 and 44–55 years old, respectively, enrolled to the study. The basic demographic data are summarized in Table II. It was found that the body mass indexes (BMI in mean + s.d.) were 24.97 ± 4.02 and 26.04 ± 7.33 kg/m^2 for healthy and patient subjects, respectively. They were considered statistically non-significantly different and slightly overweighed. Recently, the global BMI has shifted toward a higher value as the prevalence of overweight/obesity has increased by 5% every decade more rapidly in females than males (24). In addition, excess body weight has been associated with an increased risk of non-communicable diseases while female obesity was linked with poor breast cancer survival (25). Among the patient volunteers, two, five, and four subjects were at the early, middle, and last stages of ductal carcinoma, respectively. All had given birth to 1–4 children and were breastfeeding. And, 7 out of 11 were menopausal women. In terms of demographics in which disease states were ignored, it may be assumed that subjects came from a population. Thus, the endogenous composition of blood plasma disregarding those derived from breast cancer may vary homogeneously within a population's range allowing one to compare the blood plasma thermograms between normal and disease states.

DSC Thermograms and Energetics of Plasma Protein from Healthy Volunteers

Figure 2 illustrates DSC thermograms of plasma proteins of healthy volunteers. It is noted that this is an average profile of 50 individuals showing relatively high inter-individual variations (error bars in Fig. 2). Despite the variability, Fig. 2 was essentially similar to a number of normal plasma/serum thermograms previously reported (*e.g.* 7,26,27) confirming its unique characteristics. Although high abundant proteins present in blood plasma possess polymeric peptide chains as well as carry several charges, the solvophobic effect and the entropy of the chains have no influence on the final state during thermal treatment. As a consequence, the thermogram illustrates a characteristic heat transition pattern. It was noted that the thermal denaturation only produced a molten globule state followed by protein aggregation (28) which may fit very well with the simple Lumry-Eyring model utilized in the current study.

The deconvolution of a normal plasma thermogram to individually detectable protein transitions was done and

Table II. The Demographical Portrait of Female Subjects Who Enrolled in the Study

Subject	Healthy	Breast cancer
n	50	11
Age; year ($\bar{X}$ ± s.d.)	36.2 ± 10.1	49.7 ± 3.9
BW; kg ($\bar{X}$ ± s.d.)	60.9 ± 12.2	58.4 ± 9.8
Height; cm ($\bar{X}$ ± s.d.)	158.8 ± 5.6	151.2 ± 9.2

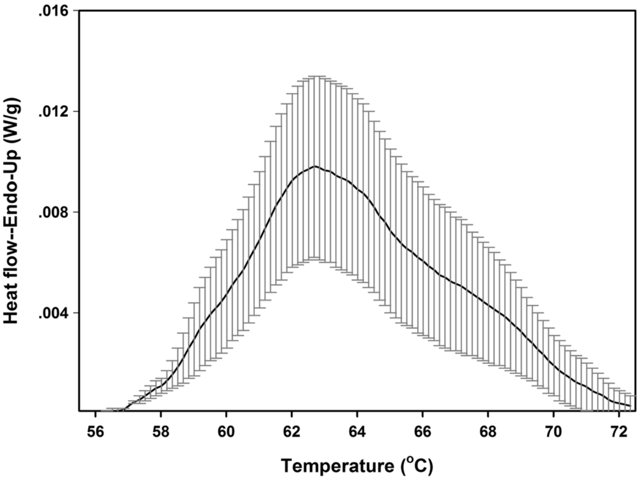

Fig. 2. The average DSC thermogram of normal plasma samples under study. The data were taken from 50 female healthy volunteers. The standard error (error bar) for each of the coordinates demonstrates the significant inter-individual variations

showed in Fig. 3a. In addition, the thermogram of albumin-enriched fraction was compared with that of parental plasma in Fig. 3b. As seen in Fig. 3a, the deconvolution revealed that there were at least four thermal transitions of abundant proteins. The findings were consistent with those reported by Koynova et al. (26). The authors indicated four transitions of most abundant plasma proteins deconvoluted out of their thermogram of blood plasma from a healthy individual with relative amount ranked in descending order as albumin, globulins, IgG/transferrin, and fibrinogen. Furthermore, it was evident that thermal transition temperature of albumin may be positioned at the midpoint of the highest amplitude of DSC tracing (*e.g.* the apex of thermograms: 7,26,29) that can be projected to correspond to Peak 2 in Fig. 2a. And, it was observed that DSC tracing of albumin-enriched fraction appeared close to Peak 2 (Fig. 2b) but slightly shifted downward showing the temperature of transition (mean ± s.d., $n = 50$) of 59.92 ± 1.49°C compared with 61.52 ± 0.49°C of Peak 2. It has been stated that a free protein always denatured at a lower temperature than ligand-bound form did (30). Also, several endogenous small molecules present in normal plasma such as fatty acids, thyroxine, bilirubin, and heme, and some metals behave as binding ligands for albumin (31). Some of these ligands might be eliminated during the process of albumin enrichment. As a result, a slight decrease in denaturing temperature was observed. Nevertheless, the difference was not statistically significant.

Apparent activation energies (E_{app}) of Peak 2 and albumin-enriched fraction were obtained from non-linear fitting of Eq. 8. It was found that E_{app} (mean ± s.d., $n = 50$) of Peak 2 and albumin-enriched fraction were 141.08 ± 18.67 and 127.79 ± 45.15 Kcal/mol, respectively. Although E_{app} values were slightly different, they were statistically non-significant. Notice also that E_{app} of albumin-enriched fraction exhibited more variation. During the enrichment, the plasma samples were diluted. It could markedly reduce the signal-to-noise ratio of the DSC tracings. As a result, the data of albumin-enriched fraction possessed relatively more variations than those of Peak 2 counterpart. Furthermore, these E_{app} values were in close agreement with that of commercial human serum albumin after the removal of stabilizing ligands

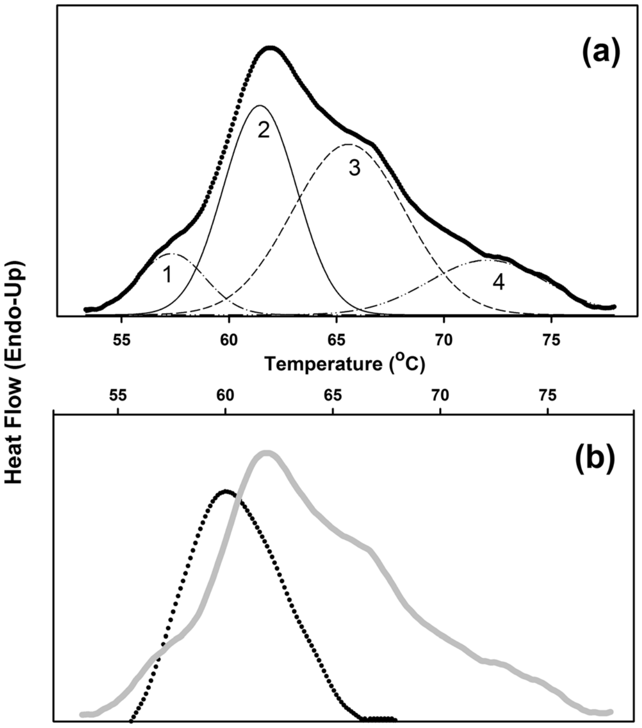

Fig. 3. a The deconvolution of a DSC endotherm of individual plasma showing at least 4 detectable protein transitions. Notice that Gaussian curves were utilized in the analysis. **b** The DSC endotherm of the albumin-enriched fraction compared with that of original plasma (gray profile)

(E_{app} value of 151.3 Kcal/mol) previously reported (19). Thus, Peak 2 confirmedly demonstrated the thermal transition of albumin in blood plasma.

DSC Thermograms of Plasma Protein from Breast Cancer Patients of Various Stages

Figure 4 displays the DSC thermograms of blood plasma from female individuals at different stages of breast cancer compared with that of a normal one (a gray curve in Fig. 4). For clarity, the corresponding Peaks 2 are also illustrated in the inset of Fig. 4. It is seen that the denaturing temperature of plasma albumin increased with the progression of the disease (Fig. 4). The temperature increasingly varied from 61.90°C for breast cancer at the early stage to 73.34°C for that at the last stage compared with 61.52 ± 0.49°C for healthy volunteers ($n = 50$). On the contrary, the albumin-enriched fractions prepared from patients' plasma did not clearly reveal the trend of temperature increment (data not shown). Again, albumin may lose its albuminomes during the process of albumin enrichment. As a result, the increased pattern of denaturing temperature was not observed.

In peptide biomarker studies, proteomic/peptidomic analyses using advanced comprehensive separations coupled with mass spectrometry (*e.g.* matrix-assisted laser desorption/ionization time-of-flight MS (29) and tandem Fourier transform MS (32)) have been primarily done on blood plasma samples. They offer specific peptide identification as well as quantification. Nevertheless, no single peptide but the changes in the pattern of a bunch of peptides emerges from the analyses as a whole reliable biomarker for such a disease (32). Those tumor peptides might turn to be albuminomes yielding the modification of stability of albumin as illustrated in Fig. 4. Similar to these findings, albumin peak of DSC thermogram was previously found to shift to a higher temperature as ovarian cancer progressed (29). It was suggested that the increased temperature was due to the disease-generated albuminome binding interaction (33) as significantly high peptide expression with upward-shifted thermograms was observed (29). It has been further evidenced that the degree of cancerous invasion was associated with the extent to which proteins had been degraded. For breast cancer, 839 distinct peptides were profiled compared with 425 peptides for healthy controls. The ratios of tumor peptide relative abundance can increasingly vary up to more than 4000-fold between breast cancer at the late stages and normal plasma (32). Also, the degradation could provide the tumor albuminomes in significant amount to markedly increase transition temperature of albumin especially at the late stage of cancerous invasion. On the other hand, other high abundant proteins such as IgG could also bind to these peptide ligands (29). But, IgG appeared as a shoulder, right after an apex (*i.e.*, albumin peak), of the thermogram (29) and its relative amount varied considerably due to the complications of disease pathologies as well as patient conditions. Thus, IgG was not chosen so as not to face the difficulties of data analysis.

The physician's conventional tumor staging (*e.g.* stage I, II, III, and IV) indicates the cancerous invasion qualitatively. Breast cancer is heterogeneous. Its key prognostic factors include primary tumor size (T), number and size of invaded regional lymph nodes (N), and the absence or presence of metastasis (M). Furthermore, these factors possess notations usually presenting as a number (*e.g.* 0, 1, 2) to describe the degrees of tumor invasion, for examples, size or extent of the tumor and existence and extent of regional lymph node involvement and whether distant metastasis is present. The higher the number, the more invasive it will be. It has been stated that the cancer staging was based on aggregate information resulting from T, N, and M category designations (22). Thus, the summation of the numbers of categories may primarily quantitate the tumor stage. The so-called tumor staging score, the summation of the numbers of notations for the key prognostic factors was plotted against the denaturing temperature of albumin in blood plasma and illustrated in Fig. 5. It turned out that the denaturing temperature was

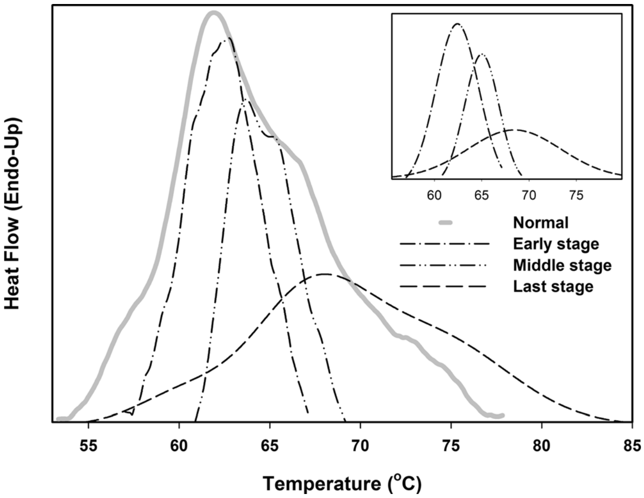

Fig. 4. The DSC thermograms of blood plasma from the individuals with different stages of breast cancer. The gray curve is thermogram of that from healthy individual. The inset shows the corresponding de-convoluted peaks out of the figure's thermograms (Peaks 2) which are expected to be albumin

linearly proportional to the staging score with a good statistical correlation (r^2 of 0.9666 presented as a dash line in Fig. 5). Presumably, the increase in denaturing temperature was directly dependent on the amount of albuminomes. Thus, the increased denaturing temperature may then imply the increase in albuminome abundance produced by the tumor invasion as breast cancer progressed.

Energetic Profile of Thermal Induced Denaturation of Plasma Albumin with Breast Cancer: a Potential Indicator for Breast Cancer Diagnosis

To have an energetic profile like Fig. 1, not only the E_{app} value derived from the thermogram, but also the concentration of ligand is needed. Quantification of peptide ligands produced by breast cancer of an individual subject was rather difficult as it required laborious and time-consuming proteomic methods of determination to do so. Fortunately, it was not necessary to quantify the peptide albuminomes as the linear relationship shown in Fig. 5 may infer the use of temperature as a representative of the amount of the tumor albuminome ligands (*e.g.* the abscissa of Fig. 1). E_{app} temperature "*energetic*" profile of thermal denaturation of albumin from blood plasma thermograms of breast cancer of various stages was constructed and showed in Fig. 6. The sigmoidal ligand binding response model illustrated in Eq. 9 was utilized as a model for statistical data fitting.

$$E_{\mathrm{app}} = E_{\min} + \frac{E_{\max} - E_{\min}}{1 + 10^{\log T_{0.5}\alpha}} \tag{9}$$

In Eq. 9, $E_{\max}$ and $E_{\min}$ are E_{app} at the extreme ends and $T_{0.5}$ and α are transition temperature when E_{app} is halfway between extreme ends and hill coefficient which describes the steepness of the fitted curve, respectively. The data were very well fitted into the model having r^2 of 0.9265. It is seen that the energetic curve in Fig. 6 apparently changed from one extreme to the other with temperature increment. And, it was found that the estimates (value ± s.e. of estimation) of E_{app} at the extreme ends were 132.68 ± 14.21 ($E_{\max}$) and 46.76 ± 8.41 ($E_{\min}$) Kcal/mol for normal (denoted as N in Fig. 6) and breast cancer at the last stage (denoted as III and IV) thermograms, respectively. In addition, the estimates of $T_{0.5}$ and α were 65.56 ± 1.16°C and -0.3491 ± 0.1455, respectively. In the case of albumin without ligand binding, it has been assumed based on Scheme 2 that the step of irreversible change was negligible for time scale at which the DSC was carried out (*e.g.* more than 1°C/min scanning rate 16); the heat associated with the protein denaturation may approach the enthalpy of unfolding (ΔH_{U}). It was previously reported that ΔH_{U} of unliganded human albumin was in the range of 88.6–115 Kcal/mol depending on the experiment settings (16,34). The energetic state of blood plasma albumin of normal healthy subjects found in the current study was comparable to ΔH_{U} previously reported confirming that the denaturation could follow Scheme 2. The presence of slight discrepancy might be due to either the differences of experimental settings or the contribution of energetics from some endogenous ligand binding (19).

During thermal induction, the protein may liberate ligands instantaneously before unfolding (35). Thus, an increased amount of tumor peptides would shift the equilibrium toward albumin-albuminome complexes yielding the increase in temperature necessitated to unfold the protein. But, the phenomenon took place regardless of the structural consequences with contribution in only slightly increased temperature (30). It might not be the case as the denaturing temperature of albumin at the late stages of cancer was far higher compared with the normal one, *e.g.*, more than 10°C increased. Upon binding, it seems that the denaturing process attained different energetic state since the decreased E_{app} leveled off at a certain point (Fig. 6). In the secondary structure, human albumin folds in a heart-shaped globule containing three helical domains indicated as domain I, II,

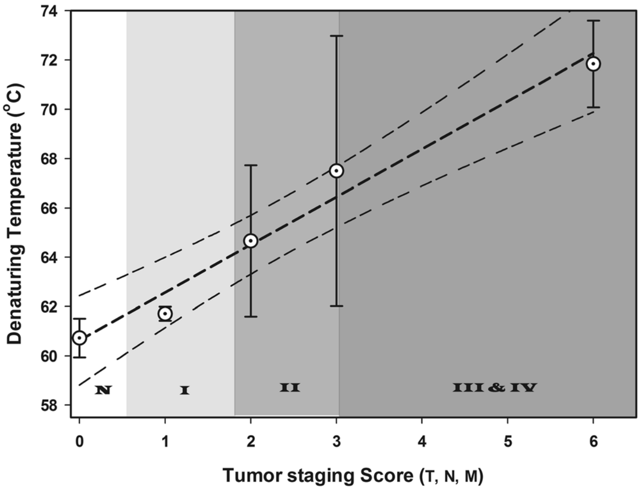

Fig. 5. The plot between primary tumor staging score and denaturing temperature of albumin of breast cancer volunteers. The shade areas indicate the staging categories (I, II, and III&IV) (the more invasions, the darker is the shade), while the area N is for normal blood plasma. The dash lines represent a linear trend and its 95% confident interval

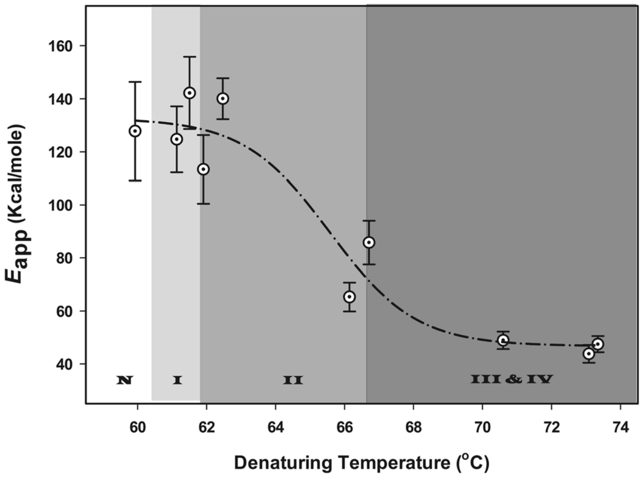

Fig. 6. Energetic profile of albumin derived from DSC thermograms of blood plasma of breast cancer of various stages. Except that of normal plasma located in area N which is the mean value with standard deviation of 50 data sets, the coordinates are the estimated values according to Eq. 8 with their standard errors of estimation presented as vertical bars. A trend line is a non-linear fitted curve base on the model of sigmoidal ligand binding response. It shows how E_{app} transit from one state to other. The shade areas indicate the staging categories (I, II, and III&IV) (the more invasions, the darker is the shade), while, the area N is for normal blood plasma

and III, each consists of sub-domains A and B. It was evident that unliganded albumin showed a remarkable difference in conformation compared with bound form (36). Among the albumin domains, sub-domain IIIA is prone to reversibly unfold due to relatively fewer interactions with other domains of the albumin molecule (37). Moreover, domain III possesses high-affinity sites (31) where the tumor albuminomes likely get associated with. It is possible that bound albuminomes might alter the albumin-albuminome complex conformation. This may cause the rate of reversible unfolding to relatively lower and become a rate-determining step driving Scheme 2 kinetically turn to Scheme 3 as indicated by 2 different energetic states seen in Fig. 6 (c.f. Figure 1). E_{app} of Fig. 6 may be the total activation energy of the weighted combination between the two depending on the presence of tumor albuminomes. Therefore, the energetic level in Fig. 6 may be used as a diagnostic parameter that might indicate the progression of breast cancer.

As seen in Fig. 6, the E_{app} value only slightly decreased at an early stage of cancerous invasion. It began to considerably decrease at the middle stage, and then flattened at the last stage. To be a good disease-related indicator, the change of E_{app} value should be observable within an early period of tumor invasion. It is because the difficulties in therapy for cancer at the late stages have been the main reason for high mortality rate. It would have been significantly reduced with prolonged life span if the disease could be diagnosed earlier. However, the magnitude of α-coefficient from data fitting was less than unity ($\alpha = -0.3491$) showing a shallow slope of the energetic curve that made the observable energetic reduction occur at the middle stage of breast cancer instead of the early one. It may be because the amount of cancerous albuminomes produced at an early stage was not sufficient to make a significant change in the energetic state during thermally induced denaturation. The determination of E_{app} value out of blood plasma thermogram might be appropriate for the disease screening rather than early diagnosis. It was previously suggested that MS-based proteomic/peptidomic techniques should be done in addition to DSC. While DSC provided a signature thermogram indicative of a disease state, MS gave the specific details of cancerous albuminomes. They were mutually complementary in the diagnosis (29). A number of earlier works claimed DSC method as a potential tool for tumor diagnosis and monitoring (*e.g.* 8,11,29). But again, they did not concretely demonstrate the potential of the machine for early diagnosis. The pitfall of DSC to early detection of the disease is still challenging.

CONCLUSION

In cancerous invasion, unusual metabolisms could provide a significant amount of small-molecule peptide albuminomes that would be able to modulate blood plasma albumin stability *via* binding interaction. It was found that the denaturing temperature of plasma albumin increased with increasing breast cancer staging score which implied the increase in cancerous albuminome abundance produced by the tumor invasion. The analysis of albumin denaturing energetics based on a rational approximation of the simple Lumry-Eyring model demonstrated that thermal denaturing transition of albumin with disease state attained different energetic level from the normal one, *i.e.*, albumin with albuminomes flavored thermal denaturation of Scheme 3 whereas unliganded one did that of Scheme 2. Increase in tumor staging resulted in albumin denaturation of Scheme 2 kinetically transform to Scheme 3. Thus, by monitoring the energetics of albumin denaturation, one could potentially diagnose and assess the prognosis of the disease. Unfortunately, the Scheme transformation was obviously observed when the tumor was not at the early stage, but the middle one. There have been the advantages of DSC including being able to use unlabeled, un-derivatized, and un-fractional samples and simple sample preparation, and a minute volume of the sample is used. But, the challenge of the use of DSC for early detection of tumor invasion still remains.

ACKNOWLEDGEMENTS

Special thanks go to PSU Scientific Equipment Center, and Drug Delivery System Excellent Center, Faculty of Pharmaceutical Sciences for Lab facilities.

FUNDING INFORMATION

The authors are grateful to the Royal Golden Jubilee Ph. D. program for partial financial support and Thailand Research Fund as well as Prince of Songkla University for the co-funding research grant.

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