(ร่าง) รายงานวิจัยฉบับสมบูรณ์

(ปกปิด)

โครงการ ความคงตัวระหว่างการเก็บรักษาของระบบนำส่งชนิดมัลติเฟส สำหรับนำส่งสารโภชนเภสัชและอาหารเพื่อสุขภาพ

โดย รองศาสตราจารย์ ดร.ปาริฉัตร หงสประภาส และคณะ

สิงหาคม 2563

สัญญาเลขที่ DBG5180024

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คณะผู้วิจัย

1. รองศาสตราจารย์ ดร.ปาริฉัตร หงสประภาส หัวหน้าโครงการ
2. นางสาวละอองดาว ว่องเอกลักษณ์ นักศึกษาปริญญาเอก
3. นางสาวสุวนุช ตันตระกูล นักศึกษาปริญญาเอก
4. นางสาวปิยธิดา เกิดช่วย ผู้ช่วยวิจัย
5. นางสาวเปรมวดี สกุลสม ผู้ช่วยวิจัย

ภาควิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร

คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

รายละเอียดโครงการ

สัญญาเลขที่ สัญญาเลขที่ DBG5180024

ชื่อโครงการ (ไทย) ความคงตัวระหว่างการเก็บรักษาของระบบนำส่งชนิดมัลติเฟสสำหรับนำส่งสารโภชนเภสัชและอาหารเพื่อสุขภาพ

ชื่อโครงการ (อังกฤษ) Storage Stability of Multi-Phase Delivery Systems for Nutraceuticals and Functional Foods

หัวหน้าโครงการ รองศาสตราจารย์ ดร.ปาริฉัตร หงสประภาส
ภาควิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร
คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์

ผู้ประกอบการผู้ร่วมทุน -

งบประมาณ 1.2 ล้านบาท ระยะเวลา 3 ปี

Abstract

This study investigated the characteristics of protein hydrolysate prepared from globular proteins, namely commercial whey protein concentrate (WPC) and mungbean protein concentrate (MPC) prepared in the laboratory, by limited hydrolysis using trypsin to increase antioxidant capacity. The antioxidative protein hydrolysates can inhibit the Maillard browning reactions of protein foods when used in sterilized protein products and in a protein powder that has undergone an accelerated transformation process at high temperatures. The ability to inhibit covalent bond formation in Maillard browning reactions of these protein hydrolysates could be used to enhance the storage stability of food protein powder.

Besides, the mixing of these protein hydrolysates with carbohydrates of different characteristics in molecular mass, water solubility, and water absorption before spray-drying could induce the molecular alignments of proteins and peptides differently before dehydration. Subsequently, the available reactive groups could be designed for specific purposes in a multi-phase delivery model close to real food systems. Using large carbohydrate molecules as excipients could control the alignments of proteins and peptides not to favor moisture-induced aggregation via covalent bond formation during storage, thus retaining the water solubility of protein hydrolysate powder although the storage time was extended.

This research also found that protein hydrolysate can be used as a carrier of asiatic acid, which is an amphiphilic compound that forms a large micelle insoluble or reduced cellular uptake when used alone. Using peptide nanocluster as a carrier of asiatic acid helped control the size of asiatic acid micelles to below 300 nm and facilitated the diffusion of asiatic acid through the cell membranes of human cell models. However, microstructural changes of protein-peptide-asiatic aggregates during digestion were observed when the multi-phase delivery systems were digested under *in vitro* simulated human system due to the digestion of proteins and carbohydrates by intestinal enzymes, the presence of bile salts, and increased osmotic pressure by small molecules of carbohydrates under digestion conditions. The fates of peptides-asiatic of submicron size bioavailability need further investigation in animal models.

Keyword Encapsulation; Protein; Hydrolysate; Stability; Mungbean; Whey

บทคัดย่อ

โปรตีนไฮโดรไลเสทจากโปรตีนเวย์ และโปรตีนถั่วเขียวซึ่งเป็นโปรตีนทรงกลมที่ผ่านการดัดแปรให้มีความสามารถในการต้านอนุมูลอิสระเพิ่มขึ้นโดยการย่อยด้วยเอนไซม์ทริพซินแบบจำกัด มีความสามารถในการยับยั้งการเกิดสีน้ำตาลแบบเมลลาร์ดของอาหารโปรตีนเมื่อนำไปใช้ในอาหารโปรตีนที่ผ่านการฆ่าเชื้อด้วยความร้อนระดับสเตอริไลซ์ และใช้ในอาหารโปรตีนชนิดผงที่ผ่านกระบวนการเร่งการเปลี่ยนแปลงที่อุณหภูมิสูง ความสามารถในการยับยั้งการเกิดพันธะโคเวเลนซ์ในการเกิดปฏิกิริยาเมลลาร์ดของโปรตีนไฮโดรไลเสทนี้ เอื้อต่อการลดการเปลี่ยนแปลงของอาหารโปรตีนชนิดผงระหว่างการเก็บรักษาได้

นอกจากนั้นแล้ว ยังพบว่าเมื่อทำแห้งโปรตีนทรงกลมที่ผ่านการย่อยแบบจำกัดดังกล่าวร่วมกับคาร์โบไฮเดรตซึ่งมีคุณสมบัติแตกต่างกันในเรื่องมวลโมเลกุล ความสามารถในการละลายน้ำ ความสามารถในการดูดน้ำและอุ้มน้ำ จะทำให้โปรตีนและเปปไทด์มีการจัดเรียงโมเลกุล (molecular alignment) และหันหมู่ที่ว่องไวต่อการเกิดปฏิกิริยา (reactive group) เข้าหากันในสารละลายก่อนการทำแห้งแตกต่างกัน การควบคุมการจัดเรียงตัวของโปรตีนและเปปไทด์ก่อนการทำแห้งนี้ช่วยให้ผู้วิจัยออกแบบคุณลักษณะของ เมทริกซ์ชนิดมัลติเฟส (multi-phase delivery system) ซึ่งใกล้เคียงกับโครงสร้างของอาหารจริงตามที่ต้องการได้ โดยการเลือกคาร์โบไฮเดรตโมเลกุลใหญ่เป็นสารกระสัย (excipient) ที่จะควบคุมให้การจัดเรียงโมเลกุลโปรตีนและเปปไทด์ไม่เอื้อต่อการเกิดพันธะโคเวเลนซ์ระหว่างการเก็บรักษาโปรตีนไฮโดรไลเสทผง และรักษาความสามารถในการละลายน้ำของโปรตีนไฮโดรไลเสทผงไว้ได้แม้อายุการเก็บของอาหารผงนานขึ้น

งานวิจัยนี้ยังพบว่า การใช้โปรตีนไฮโดรไลเสทเป็นสารพากรดเอเชียติกซึ่งปกติแล้วเป็นสารแอมฟิไฟล์และสามารถเกิดไมเซลล์ (micelle) ขนาดใหญ่จนไม่ละลายน้ำหรือดูดซึมเข้าเซลล์ ให้ถูกดูดซึมเข้าสู่เซลล์ได้ โดยการทำให้โปรตีนหรือเปปไทด์เกิดปฏิกิริยากับกรดเอเชียติก (Asiatic acid) ด้วยอันตรกิริยาไฮโดรโฟบิก (hydrophobic interactions) ส่งเสริมให้กรดเอเชียติกถูกดูดซึมเข้าเซลล์ทดสอบได้อย่างมีประสิทธิผล มากกว่าการใช้กรดเอเชียติกอย่างเดียว บ่งชี้ว่าการใช้เปปไทด์นาโนคลัสเตอร์ (peptide nanocluster) ในการเป็นสารพากรดเอเชียติก ช่วยควบคุมขนาดของไมเซลล์กรดเอเชียติกให้ต่ำกว่า 300 นาโนเมตรและเอื้อต่อการที่กรดเอเชียติกจะแพร่ผ่านผนังเซลล์และออกฤทธิ์เมื่อทำการทดสอบในโมเดลเซลล์มนุษย์ อย่างไรก็ตาม การเปลี่ยนแปลงของโครงสร้างระดับจุลภาคของโปรตีนและกรดเอเชียติกระหว่างการย่อยอาหารยังเกิดขึ้นได้ต่อภายใต้สรีรวิทยาการย่อยในระบบจำลองของมนุษย์ เนื่องมาจากการย่อยโปรตีนและคาร์โบไฮเดรตด้วยเอนไซม์ในลำไส้เล็ก เกลือน้ำดี และแรงดันออสโมติกที่เพิ่มขึ้นภายใต้สภาวะการย่อยที่มีโมเลกุลขนาดเล็กเกิดขึ้น ทำให้อนุภาคของเปปไทด์ และโอลิโกแซคคาไรด์ รวมทั้งกรดเอเชียติกมีการเปลี่ยนแปลงในเรื่องขนาดและความสามารถในการถูกดูดซึม ซึ่งควรได้รับการศึกษาต่อไปในระดับสัตว์ทดลอง

คำสำคัญ .การห่อหุ้ม โปรตีน ไฮโดรไลเสท ความคงตัว เวย์ ถั่วเขียว ทำแห้ง

บทสรุปผู้บริหาร

การผลิตอาหารเชิงฟังค์ชันหรืออาหารเพื่อสุขภาพที่มีการเติมสารออกฤทธิ์ทางชีวภาพนั้น มีความแตกต่างจากการผลิตสารโภชนเภสัชที่ใช้เป็นผลิตภัณฑ์เสริมอาหาร ทั้งนี้ เพื่อให้อาหารเชิงฟังค์ชันยังคงคุณค่าทางโภชนาการและเป็นแหล่งของสารออกฤทธิ์ที่คงตัวระหว่างการแปรรูป การเก็บรักษา สารออกฤทธิ์สามารถถูกดูดซึมและส่งเสริมสุขภาพภายหลังการกินร่วมกับสารอาหารอื่น การย่อยในระบบทางเดินอาหาร และการดูดซึมร่วมกับสารอาหารเข้าสู่เซลล์และการมีประสิทธิผล

งานวิจัยนี้ จึงให้ความสำคัญแก่การทำความเข้าใจปฏิกิริยาระหว่างสารอาหารกับสารออกฤทธิ์ทางชีวภาพในระหว่างกระบวนการผลิต การเก็บรักษา และการทดสอบการออกฤทธิ์ภายหลังการย่อย โดยทำการทดสอบในโมเดลเซลล์มนุษย์ ทำการเลือกโปรตีนทรงกลมที่ผ่านการดัดแปรด้วยเอนไซม์ทริพซินเป็นโมเดลของสารอาหารโปรตีนที่ให้คุณสมบัติต้านอนุมูลอิสระและยับยั้งการเกิดปฏิกิริยาเมลลาร์ดหรือไกลเคชัน เพื่อทำหน้าที่เป็นสารออกฤทธิ์ทางชีวภาพจากสารอาหารโดยตรงนอกเหนือจากการเป็นแหล่งพลังงานและกรด แอมิโนจำเป็น โปรตีนทรงกลมที่ถูกย่อยนี้ ยังสามารถทำหน้าที่เป็นสารพา (carrier) สารออกฤทธิ์ชนิดต้านมะเร็ง (anticancer compound) จากใบบัวบกซึ่งมีโครงสร้างเป็น pentacyclic triterpene คือกรดเอเชียติก (asiatic acid) ซึ่งไม่ละลายน้ำและไม่ละลายในน้ำมันเข้าสู่เซลล์

งานวิจัยนี้ได้ทำการออกแบบเมทริกซ์ชนิดมัลติเฟสเพื่อนำส่งสารออกฤทธิ์ที่ไม่ละลายน้ำและไม่ละลายในน้ำมัน และมีคุณสมบัติแอมฟิไฟล์ โดยกำหนดชนิดของคาร์โบไฮเดรตที่จะควบคุมการจัดเรียงตัวของสารออกฤทธิ์และสารพาไว้ในโครงสร้างอสัณฐานของคาร์โบไฮเดรตได้ โดยเลือกชนิดของคาร์โบไฮเดรตในรูปของน้ำตาลไดแซคคาไรด์ มอลโทเดกซ์ทริน และสตาร์ชในสัดส่วนที่เหมาะสม สำหรับกระบวนการทำแห้งแบบพ่นฝอยเพื่อให้อาหารผงที่ดังกล่าวมีความคงตัวระหว่างการเก็บรักษา

สารบัญ

สารบัญภาพ

สารบัญตาราง

บทที่ 1 บทนำ

1.1 ความเป็นมาของโครงการ (Research rationale)

การใช้ประโยชน์จากสารออกฤทธิ์ทางชีวภาพ (bioactive compounds) เมื่อบริโภคเป็นผลิตภัณฑ์เสริมอาหาร (food supplement) หรือสารโภชนเภสัช (nutraceuticals) กับการบริโภคสารออกฤทธิ์ร่วมกับอาหารโดยตรงในอาหารเพื่อสุขภาพหรืออาหารเชิงฟังค์ชัน (health food หรือ functional food) มีความแตกต่างกันในเรื่องประสิทธิผลของสารออกฤทธิ์ เนื่องจากความแตกต่างในเรื่องของสัดส่วนระหว่างสารออกฤทธิ์กับสารอาหารที่อยู่ในสูตร เพราะสารอาหารสามารถทำปฏิกิริยากับสารออกฤทธิ์ ซึ่งอาจส่งเสริม หรืออาจชะลอการดูดซึมสารออกฤทธิ์เข้าสู่เซลล์ ในการผลิตสารโภชนเภสัชตามแนวทางของเภสัชวิทยานั้น มักมีการเติมสารกระสัย (excipient) ที่เฉื่อยต่อการเกิดปฏิกิริยากับสารออกฤทธิ์ในระหว่างการเก็บรักษาและไม่มีสารอาหารอื่นอยู่ในสูตร เพื่อให้สารออกฤทธิ์ส่งผลตามปริมาณที่กำหนด (dose) โดยไม่ถูกรบกวนการออกฤทธิ์ (interfere) ด้วยสารอาหาร ซึ่งอาจเห็นได้ว่า การบริโภคสารโภชนเภสัชในรูปผลิตภัณฑ์สำเร็จไม่ว่าจะอยู่ในรูปของเหลว เป็นผง หรืออัดเม็ด อาจมีข้อแนะนำให้บริโภคก่อนหรือหลังอาหาร

อย่างไรก็ตาม การผลิตอาหารเชิงฟังค์ชันหรืออาหารเพื่อสุขภาพที่มีการเติมสารออกฤทธิ์ทางชีวภาพนั้น มีความแตกต่างจากการผลิตสารโภชนเภสัชดังกล่าวข้างต้นโดยสิ้นเชิง เพื่อให้อาหารเชิงฟังค์ชันยังคงคุณค่าทางโภชนาการ และเป็นแหล่งของสารออกฤทธิ์ที่สามารถดูดซึมและส่งเสริมสุขภาพภายหลังการกิน การย่อยในระบบทางเดินอาหาร และการดูดซึมร่วมกับสารอาหารเข้าสู่เซลล์และการมีประสิทธิผล

งานวิจัยนี้ จึงให้ความสำคัญแก่การทำความเข้าใจปฏิกิริยาระหว่างสารอาหารกับสารออกฤทธิ์ทางชีวภาพ โดยเลือกโปรตีนทรงกลมที่ผ่านการย่อยด้วยเอนไซม์ทริพซินเป็นโมเดลของสารอาหารโปรตีนที่ให้คุณสมบัติต้านอนุมูลอิสระและยับยั้งการเกิดปฏิกิริยาเมลลาร์ดหรือไกลเคชัน (glycation) ในอาหาร เพื่อทำหน้าที่เป็นสารออกฤทธิ์ทางชีวภาพจากสารอาหารโดยตรงนอกเหนือจากการเป็นแหล่งพลังงานและกรดอมิโนจำเป็น โปรตีนทรงกลมที่ถูกย่อยนี้ ยังสามารถทำหน้าที่เป็นสารพา (carrier) สารออกฤทธิ์ชนิดต้านมะเร็ง(anticancer compound) สกัดจากใบบัวบกซึ่งมีโครงสร้างเป็น pentacyclic triterpene คือกรดเอเชียติก (asiatic acid) ซึ่งไม่ละลายน้ำและไม่ละลายในน้ำมันเข้าสู่เซลล์

การเข้าใจปฏิกิริยาที่เกิดขึ้นจะช่วยให้ผู้วิจัยออกแบบเมทริกซ์ชนิดมัลติเฟสเพื่อนำส่งสารออกฤทธิ์ที่ไม่ละลายน้ำและไม่ละลายในน้ำมัน และมีคุณสมบัติแอมฟิไฟล์ โดยกำหนดชนิดของคาร์โบไฮเดรตที่จะควบคุมการจัดเรียงตัวของสารออกฤทธิ์และสารพาไว้ในโครงสร้างอสัณฐานของคาร์โบไฮเดรตได้ โดยเลือกชนิดของคาร์โบไฮเดรตในรูปของน้ำตาลไดแซคคาไรด์ มอลโทเดกซ์ทริน และสตาร์ชในสัดส่วนที่

เหมาะสม สำหรับกระบวนการทำแห้งแบบพ่นฝอยเพื่อให้อาหารผงที่ดังกล่าวมีความคงตัวระหว่างการเก็บรักษา

1.2 วัตถุประสงค์ (Objectives)

1. เพื่อประมวลองค์ความรู้พื้นฐานในการรักษาความคงตัวของอาหารเพื่อสุขภาพชนิดผงที่มีโปรตีนทรงกลม (globular proteins) ได้แก่ โปรตีนเวย์ และโปรตีนถั่วเขียว และคาร์โบไฮเดรตเป็นพอลิเมอร์ห่อหุ้มสารออกฤทธิ์ทางชีวภาพ เนื่องมาจากความไม่เข้ากันทางอุณหพลศาสตร์ (thermodynamic incompatibility) ของโปรตีนและคาร์โบไฮเดรต ซึ่งจะทำให้เกิดการแยกเฟสระดับจุลภาค (microstructural phase separation) ระหว่างขั้นตอนการผลิตอาหารผง การเก็บรักษาอาหารผงที่มีสารโภชนเภสัชในกลุ่มที่มีขั้ว มีประจุ และกลุ่มที่ไม่มีขั้ว และการย่อยอาหารผงที่นำมาละลายน้ำในระบบจำลอง
2. ทำการทดสอบประสิทธิผลของการห่อหุ้มสารโภชนเภสัชชนิดที่ไม่ละลายน้ำด้วยระบบมัลติเฟส (multiphase encapsulation) ในโมเดลเซลล์มนุษย์ (human cell model) เมื่อใช้มอลโทเดกซ์ทริน สตาร์ช หรือน้ำตาลไดแซคคาไรด์ เป็นแหล่งคาร์โบไฮเดรตซึ่งจะให้อุณหภูมิในการเปลี่ยนสถานะกลาส (glass transition temperature) ของตัวอย่างอาหารผงสูงกว่า 60 °ซ ใช้เปบไทด์นาโนคลัสเตอร์ของโปรตีนถั่วเขียว (mungbean peptide nanocluster) เป็นสารพา (carrier) และสารไตรเทอร์ปีนส์กรดเอเชียติก (asiatic acid) ซึ่งมีคุณสมบัติต้านมะเร็ง เป็นสารโภชนเภสัชทดสอบ

1.3 แผนการดำเนินงาน (Research methodology)

- ทำการผลิตโปรตีนไฮโดรไลเสทต้านอนุมูลอิสระชนิดผงจากโปรตีนเวย์เข้มข้นเชิงการค้า และโปรตีนถั่วเขียวเข้มข้นที่เตรียมในห้องปฏิบัติการ โดยทำการย่อยโปรตีนด้วยเอนไซม์ทริพซิน
- โปรตีนไฮโดรไลเสทจะถูกทำแห้งแบบพ่นฝอยร่วมกับสารกระสัยที่เป็นคาร์โบไฮเดรตเพื่อตรึงโปรตีนและเปบไทด์ในสภาวะกลาสของคาร์โบไฮเดรต ซึ่งมี glass transition temperature ไม่ต่ำกว่า 60 °ซ ภายหลังกระบวนการทำแห้ง ยืดอายุการเก็บของผลิตภัณฑ์ชนิดผง
- ทำการทดสอบการนำไปใช้ประโยชน์เป็นสารลดการเกิดสีน้ำตาลจากกระบวนการให้ความร้อน และการใช้เป็นสารพาสารต้านมะเร็งในกลุ่มของไตรเทอร์ปีนส์

บทที่ 2 การผลิตและการใช้ประโยชน์โปรตีนไฮโดรไลเสทต้านอนุมูลอิสระจากโปรตีนเวย์ที่ถูกตรึงในสารกระสัยคาร์โบไฮเดรตชนิดต่างๆ ระหว่างการทำแห้งแบบพ่นฝอย

2.1 Lowering the Maillard reaction products (MRPs) in heated whey protein products and their cytotoxicity in human cell models by whey protein hydrolysate

ABSTRACT

This study investigated the potential use of reconstituted whey protein hydrolysate as an antibrowning agent in thermally processed foods and as a chemopreventive ingredient in biological systems. Hydrolysates were prepared by tryptic (EC 3.4.21.4) hydrolysis of whey protein concentrate (WPC) or heated (80 °C for 30 min) whey protein concentrate (HWPC). Tryptic hydrolysis of WPC and HWPC increased the oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) antioxidant capacity from 0.2 to 0.5 μmol Trolox equivalent (TE)/mg protein in both whey protein hydrolysate (WPH) and heated whey protein hydrolysate (HWPH) (P<0.05). The reconstituted WPH and HWPH could prevent the formation of Maillard reaction products (MRPs) induced by a thermal process employed on WPC suspensions between 80 to 121 °C in the presence of lactose up to 0.25 M (P<0.05). The MRPs in HWPC were cytotoxic to both normal human intestinal FHs 74 Int cells and human epithelial colorectal carcinoma Caco-2 cells. The IC_{50} of HWPC was around 3.18–3.38 mg/mL protein. Nonetheless, when both cell types were grown in media supplemented with WPH and HWPH prior to the uptake of MRPs in HWPC at 3.5 mg/mL, they were able to survive (P<0.05). Overall, this study indicated the efficacy of WPH and HWPH in the prevention of MRP cytotoxicity. It was suggested that the $ORAC_{FL}$ antioxidant capacity of WPH and HWPH needed to be high enough to provide a chemopreventive effect against MRP cytotoxicity.

1. Introduction

Whey proteins are defined as the proteins remaining in milk serum after the removal of caseins (Farrell, 1980). They include β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin, etc. Different methods are employed to concentrate and purify whey proteins to be used as functional food ingredients or nutraceuticals (Morr & Ha, 1993). Whey protein products, including whey protein concentrate (WPC) and whey protein isolate (WPI), are commonly used as food ingredients: as emulsifying, foaming or gelling agents, and as dietary supplements.

The high content of the sulfur-containing amino acids cysteine and methionine, which can enhance immune function through intracellular conversion to glutathione, has driven interest in the use of whey proteins in medical treatments in recent years. The biological activities of whey proteins in the prevention and treatment of cancer have been investigated extensively (Bounous, 2000; Bounous & Molson, 2003). The anticancer and immunomodulatory activities were focused on undenatured WPI and WPC in order to preserve the cysteine-rich conformation (Bounous & Gold, 1991; Bounous, 2000; Tsai, Chang, Chen, & Lu, 2000; Bounous & Molson, 2003).

However, the reduction in such biological activities of whey protein in food products due to the loss of cysteine is inevitable if WPC or WPI are to be used as food ingredients in processed foods. For example, heat treatment employed on WPI as a cold-setting agent in meat products (Hongsprabhas & Barbut, 1999) could reduce the cysteine content of WPI due to disulfide bond formation during heat treatment (Hongsprabhas & Barbut, 1997). Thermal alteration of the SH group in the cysteine residue of WPC and the ε-amino group in lysine residue, which contain residual lactose greater than that found in WPI, could occur via glycation during heat treatment known as Maillard reaction (Gerrard, 2006; Loveday, Hindmarsh, Creamer, & Singh, 2010). Therefore, the complexity of the interactions among food constituents during food processing and storage may lead to difficulties in preserving the biological activities of therapeutic whey proteins.

Generally, Maillard reactions are essential in introducing appealing flavors and colors in foods. However, Maillard reactions could cause the loss of nutritive value. Additional negative influences of Maillard reaction products (MRPs) on human health are the formation of mutagenic and carcinogenic compounds if consumed in the diet (Gerrard, 2006). Nevertheless, recent investigations indicated that the MRPs from reducing sugar–amino acid models exhibited antioxidant capacity (Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001; Mesa, Silván, Olza, Gil, & del Castillo, 2008). However, only a few research groups have considered both the positive and negative effects of MRPs generated during normal food processing in biological systems.

The influence of heat treatment on chemical and biological characteristics of WPC thus was explored in this study. We have addressed the influences of MRPs on antioxidant capacity in parallel with cell cytotoxicity of the heated WPC formed by thermal processing at pasteurization and sterilization levels. In addition, attempts have been made to investigate the potential use of whey protein hydrolysate (WPH) obtained from tryptic hydrolysis in the prevention of brown pigment formation in thermally processed WPC suspensions, and in the lowering of the cytotoxicity of thermally denatured WPC. We hypothesized that the use of antioxidative WPH could prevent thermally induced MRP formation and cytotoxicity of heated WPC. This was based on the fact that food-derived peptides with biological activities (e.g., cyto- or immunomodulatory and/or

antioxidative activities) could be obtained during enzymatic hydrolysis of milk, egg, fish, meat and soy proteins (Hartmann and Meisel, 2007).

2. Materials and methods

Commercial WPC was imported and repacked by a local distributor in Thailand. It contained 75.83% protein (wet basis [wb]), 6.5% moisture content (wb), 2.71% ash (wb), 1.05% fat (wb) and 13.91% carbohydrate (wb) (AOAC, 2000). Trypsin (EC 3.4.21.4; 10,000 BAEE Umg^{-1}) and fluorescein (Na salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Trolox (6–hydroxy-2,5,7,8-tetramethylchroman–2–carboxylic acid), potassium persulfate and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Cassava maltodextrin with dextrose equivalent (DE) of 10 (Neo-Maldex®, Neotech Food Co., Bangkok, Thailand) and trehalose (kindly provided by the East Asiatic Company Ltd., Bangkok, Thailand) were used as carbohydrate excipients during spray-drying.

2.1. Characteristics of heated whey protein concentrate (HWPC)

2.1.1. Brown pigment formation

WPC suspensions (2% protein w/v) were prepared in distilled water, heated at 40, 50, 60, 70, 80 and 90 °C for 30 min, cooled to room temperature (25 °C) and evaluated for brown pigment using a diluted 0.08% protein (w/v) suspension by measuring the absorbance at 420 nm with a UV-Visible spectrophotometer (Spectronic GENESYS 10; Thermo Fisher Scientific, Waltham, MA) and corrected for turbidity by subtracting the absorbance at 620 nm using the method described by Pan & Melton (2007).

2.1.2. Antioxidant capacity

WPC suspensions (2% protein w/v) were prepared in distilled water, heated at 40, 50, 60, 70, 80 and 90 °C for 30 min, cooled to room temperature (25 °C) and evaluated by oxygen radical absorbance capacity assay determined by fluorescein ($ORAC_{FL}$) (Prior et al., 2003; Dávalos, Gómez-Cordovés, & Bartolomé, 2004) in comparison to the unheated WPC suspensions. Briefly, the $ORAC_{FL}$ assays were carried out on a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). The reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4); the final reaction mixture was 200 μL. Antioxidant (20 μL; Trolox 10–90 μM or sample 0.1 mg/mL) and fluorescein (120 μL; 700 nM, final concentration) were loaded into a black 96-well microplate reader which was programmed to record the fluorescence of fluorescein on every cycle. During cycle 4, a pump was programmed

to inject 60 μL of AAPH into the respective wells to give a final AAPH concentration of 12 mM. The plate contents were mixed by shaking for 8 s. The temperature of the incubator was set to 37 °C, and a fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was used.

The final $ORAC_{FL}$ values were calculated by using a linear equation between the Trolox standards or sample concentration and net areas under the fluorescein decay curves. The area under curve (AUC) was calculated as:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 +, ..., + f_i/f_4) \times CT$$

where f_4 was the initial fluorescence reading at cycle 4, f_i was fluorescence reading at cycle i, and CT was the cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of a sample. The $ORAC_{FL}$ values of samples were then reported as μmol Trolox equivalent per mg of protein (μmol TE/mg protein).

2.1.3. Spray-drying of whey proteins in different carbohydrate excipients

WPC suspension (5.69 % protein w/v) was prepared in distilled water and divided into two fractions. The first one was mixed with carbohydrate excipients – namely cassava maltodextrin (MD) having dextrose equivalent (DE) of 10, or trehalose (TH), using the ratio between WPC to carbohydrate excipient of 0.3:0.7 – and spray-dried (Mini Spray Dryer B-190; Buchi, Flawil, Switzerland). The suspension (20% total solids) was fed at 4.0 ± 0.5 mL/min and dried using inlet air of 130 °C and outlet air of 100 °C, with a flow rate of drying air around 600 m^3/min. This fraction was designated as WPC powder. The second fraction was heated at 80 °C for 30 min, cooled to room temperature (25 °C), mixed with carbohydrate excipients, and spray-dried. This fraction was called HWPC. Both WPC and HWPC powder were stored at -20 °C prior to reconstitution with distilled water. They were analyzed for brown pigment formation and $ORAC_{FL}$ antioxidant capacity using the methods described above.

2.1.4. Cell cytotoxicity of WPC and HWPC spray-dried powder

Human hepatoblastoma HepG2 (ATTC HB-8065), epithelial colorectal carcinoma Caco-2 (ATCC HTB-37), and normal small intestine FHs 74 Int (ATCC CCL-241) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MA). HepG2 or Caco-2 cells were plated in a 96-well plate at an initial density of 2 x 10^4 cells/well with Eagle's minimum essential medium (MEM; Gibco®, Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (FBS; HyClone, Logan, UT). FHs 74 Int cells were plated in a 96-well plate at an initial density of 2.5 x 10^4 cells/well with ATCC Hybri-Care medium (Manassas, VA) supplemented with 10 μg/mL human epidermal growth factor (EGF) (Gibco, Invitrogen). The FBS used for Caco-2 was 20%, while that used for HepG2 and FHs 74 Int was 10%, in accordance with ATCC recommendations. Media were filtered through a 0.2 μm polystyrene filter (Corning, Corning, NY). The cells were grown at 37 °C under 5.0% CO_2 atmosphere. Twenty-four hours after seeding, the media were replaced with new media supplemented with predetermined doses of WPC or HWPC to obtain 0–4 mg whey protein/mL, and grown for 48 h to determine the 50% inhibition concentration (IC_{50}) of WPC and HWPC in each cell model. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen) staining method (Moll & Zaika, 2001), using a Bio-Rad model 550, microplate reader (Hercules, CA).

2.2. Use of whey protein hydrolysate (WPH) and heated whey protein hydrolysate (HWPH) to reduce brown pigment formation and cell cytotoxicity of heated whey protein concentrate (HWPC)

2.2.1. Preparation of whey protein hydrolysate and whey protein hydrolysate powder

One hundred seventy-one g of WPC or HWPC suspension (5.69% protein w/w in distilled water) was added with 1 mL of 97.32 mg trypsin (EC 3.4.21.4) to obtain a protein to trypsin ratio of 100:1. The pH was adjusted to 8.0 with 1 M NaOH. Hydrolysis was allowed to proceed for 1 h at 37 °C; trypsin was inactivated by adjusting the pH to 2.0 using 0.1 M HCl. The hydrolysate (WPH or HWPH) suspensions were then mixed with TH excipient using a ratio between WPH (or HWPH) to excipient of 0.3:0.7. The mixed suspensions were spray-dried using a Mini Spray Dryer B-190 (Buchi) using the method described above. The reconstituted WPH and HWPH were characterized for brown pigment formation and $ORAC_{FL}$ antioxidant capacity.

2.2.2. Effect of whey protein hydrolysate addition on brown pigment formation of thermally processed whey protein concentrate

WPH or HWPH powder was added to WPC suspension (1% protein w/v) in 0.1 M phosphate buffer, pH 8.0, and heated at 80 °C for 30 min, 90 °C for 30 min, or 121 °C for 15 min. The protein content was 1.3% w/v in the final WPC+WPH or WPC+HWPH suspensions. After thermal processing at different heating schemes, the mixed suspensions were diluted with 0.1 M phosphate buffer, pH 8.0, to obtain the 0.08% protein w/v solutions that were determined for brown pigment formation, using the method as described above.

2.2.3. Effect of whey protein hydrolysate addition on the survival of human cell lines

Caco-2 cells were plated in a 96-well plate at an initial density of 2×10^4 cells/well with MEM supplemented with FBS for 24 h using the method described above. After seeding, the medium was replaced with new medium with predetermined added doses of WPH or HWPH. Cell viability was evaluated by MTT assay as described above. FHs 74 Int cells were plated in a 96-well plate at an initial density of 2.5×10^4 cells/well with Hybri-Care supplemented with 10 µg/mL EGF and 10% FBS, as described above.

In *control treatment*: 24 h after initial seeding, the medium was replaced with new medium and the cells were grown for 72 h; then the medium was replaced again, and the cells were grown for another 48 h. In *pre-treatment with WPH*: 24 h after seeding, the medium was replaced with new medium containing 2.5 mg/mL WPH; the cells were then grown for 72 h. After 72 h, the medium was replaced with new medium with 3.5 mg/mL HWPC added; the cells were then grown for another 48 h. In *post-treatment with WPH*: 24 h after seeding, the medium was replaced with new medium containing 3.5 mg/mL HWPC; the cells were then grown for 48 h. After 48 h, the medium was replaced with new medium with 2.5 mg/mL WPH added; the cells were then grown for another 72 h. Cell viability in each treatment was evaluated by an MTT test.

2.2.4. Statistical analyses

Experiments were carried out in two separate trials of spray-drying; each trial was run in triplicate. The data were analyzed by analysis of variance (ANOVA) with significance at $P<0.05$. Significant differences among mean values were determined by Duncan's multiple range test. All statistical analyses were performed using SPSS software Version 12.

3. Results and discussion

3.1. Characteristics of heated whey protein concentrate (HWPC)

The $ORAC_{FL}$ antioxidant capacity of HWPC was significantly lowered from 0.221 to 0.125 μmol TE/mg protein after heat treatment at a temperature above 80 °C for 30 min (P<0.05; **Fig. 1**). The reduction in $ORAC_{FL}$ antioxidant capacity of WPC coincided with the increase in brown pigment formation, measured as OD_{420} of 2% protein w/v WPC suspensions heated from 27 °C to 90 °C. Both alterations occurred at temperatures above the denaturation temperature of whey proteins: i.e. 62–68 °C for α-lactalbumin and 78–83 °C for β-lactoglobulin, the two major proteins in whey (Morr & Ha, 1993).

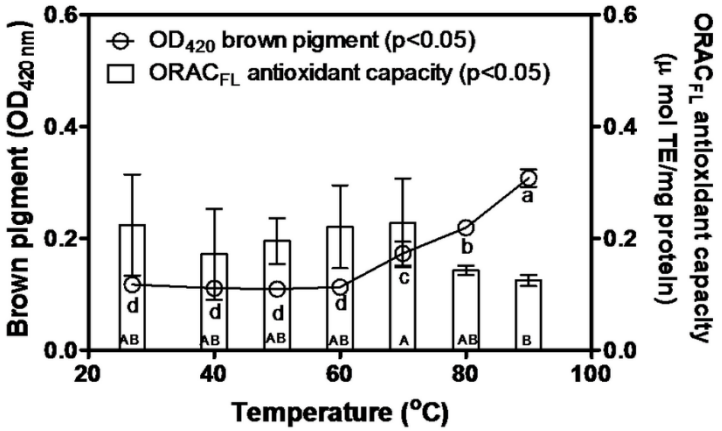

Fig. 1 Effect of heat treatment for 30 min at different heating temperatures on brown pigment formation measured as OD_{420nm} and $ORAC_{FL}$ antioxidant capacity. Bars represent standard deviation.

Nevertheless, co-drying of WPC in either cassava maltodextrin or trehalose excipients, at a protein to carbohydrate ratio of 0.3:0.7, did not alter brown pigment level and antioxidant capacity ($P \geq 0.05$, **Fig. 2**) compared to those of WPC suspensions before drying. Reconstituted HWPC dried in both carbohydrate excipients, however, had higher brown pigment than HWPC before drying (P<0.05, **Fig. 2a**). This was probably because the MRPs formed during heat treatment at 80 °C for 30 min underwent an advanced stage of glycation. Nevertheless, HWPC co-dried in both carbohydrate excipients showed higher antioxidant capacity compared to the samples before drying (P<0.05).

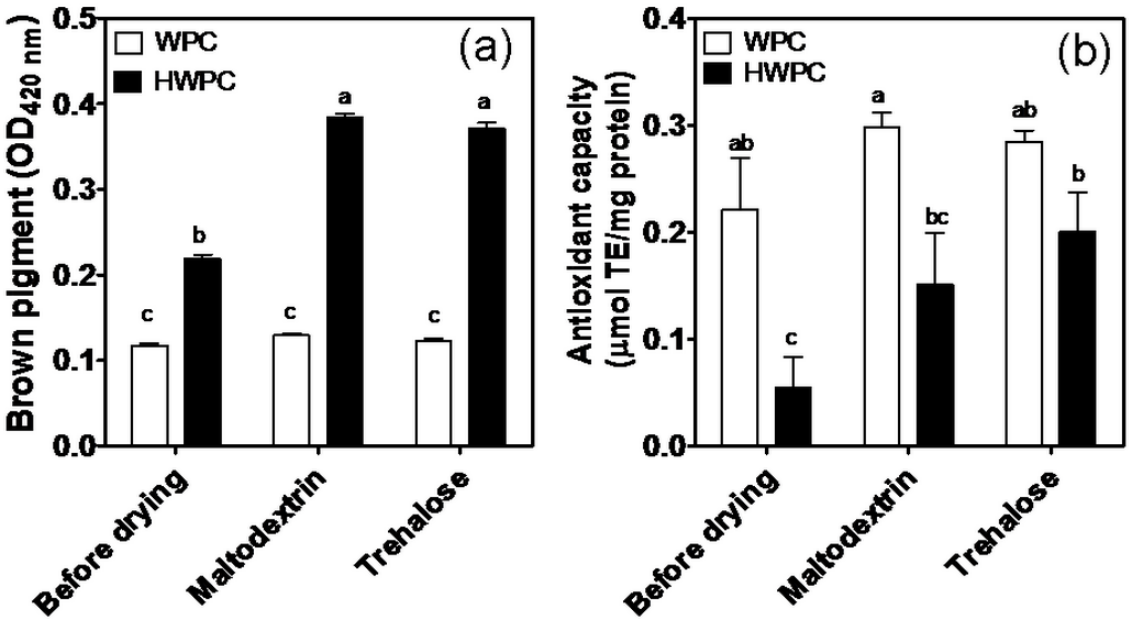

Fig. 2Effect of spray-drying on (a) brown pigment formation and (b) $ORAC_{FL}$ antioxidant capacity of reconstituted whey protein concentrate (WPC) and heated whey protein concentrate (HWPC). Bars represent standard deviation.

The addition of reconstituted WPC at levels of 0–2.5 mg protein/mL to normal media did not significantly affect human hepatoblastoma HepG2 and colorectal carcinoma Caco-2 cells (**Fig. 3**). Although the reconstituted WPC and HWPC had slightly higher antioxidant capacity compared to the samples before drying, HWPC showed cytotoxicity on human epithelial colorectal carcinoma Caco-2 cells when the concentration of HWPC was added up to 2.0 mg protein/mL. Cell survival declined to almost 60% at 2.5 mg protein/mL. This might be due to the toxicity from MRPs generated during heat treatment at 80 °C for 30 min in HWPC. The carbohydrate excipients present had no effect on the survival of both cell types ($P \geq 0.05$), although the media could be hypertonic for human cell lines.

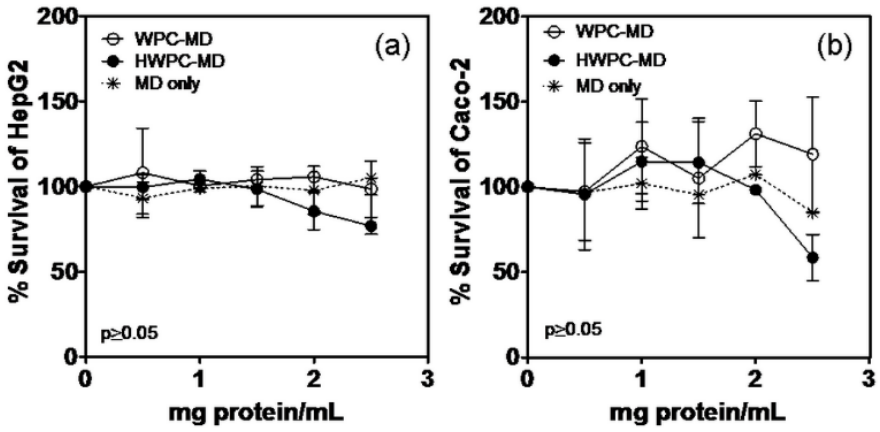

Fig. 3Effect of reconstituted whey protein concentrate (WPC) and heated whey protein concentrate (HWPC) addition on survival of (a) HepG2 and (b) Caco-2 cell models. Both WPC and HWPC were co-dried in trehalose (TH) using a spray-dryer. Dotted line represents the effect of TH excipient existing in WPH and HWPH powders at corresponding concentrations. Bars represent standard deviation.

Nevertheless, the cytotoxicity of HWPC was not evident in human hepatoblastoma HepG2 cells ($P \geq 0.05$). This suggested that Caco-2 cells were more sensitive to cytotoxicity from MRP in HWPC than were HepG2. Results also suggested that an antioxidant capacity value of around 0.2 μmol TE/mg protein was not high enough to prevent cell damage in Caco-2 cells caused by MRPs in HWPC.

Generally, hepatic cells are ideal for studying chemopreventive potentials of nutraceuticals due to their effective detoxifying functions (Goya, Martin, Ramos, Mateos, & Bravo, 2009). Considering that the MRPs need to be absorbed through intestinal cells prior to the entering into blood circulation and subsequently to the liver, only Caco-2 and FHs 74 Int were further investigated for cytotoxicity of HWPC at higher concentrations. An increase in the concentration of HWPC up to 4.0 mg protein/mL significantly reduced the survival of Caco-2 and FHs 74 Int cells ($P<0.05$). IC_{50} values of HWPC were determined as 3.18 mg protein/mL for Caco-2, and 3.38 mg protein/mL for FHs 74 Int ($P \geq 0.05$).

It is likely that the MRPs formed during heat treatment at 80 °C for 30 min, together with the reduction of antioxidant capacity, played a significant role in the cytotoxicity of Caco-2 and FHs 74 Int cells. Apparently the presence of MRPs in HWPC at concentrations of HWPC above 2.0 mg protein/mL was cytotoxic to both cancer cells and normal cells. This suggested that some measures are required to reduce the risk of MRP cytotoxicity. This is because the protein concentration commonly found in diets, according to the recommended daily allowance (RDA), is generally higher

than the protein concentration investigated in this study. Normal cooking methods could generate the risk of MRPs forming under some heating conditions.

3.2. Use of whey protein hydrolysate (WPH) and heated whey protein hydrolysate (HWPH) to reduce brown pigment formation and cell cytotoxicity of heated whey protein concentrate (HWPC)

WPC subjected to heat treatment at 80 °C for 30 min had increased brown pigment formation, an indicator for MRPs, and may be cytotoxicity to human cells. Fig. 4 illustrates that the MRPs induced by heat treatment could be reduced in the presence of WPH and HWPH ($P<0.05$). The addition of 0.3% protein from reconstituted WPH or HWPH prior to heat treatments of WPC lowered the brown pigment formation ($P<0.05$) at different lactose concentrations up to 0.25 M under various heating schemes investigated.

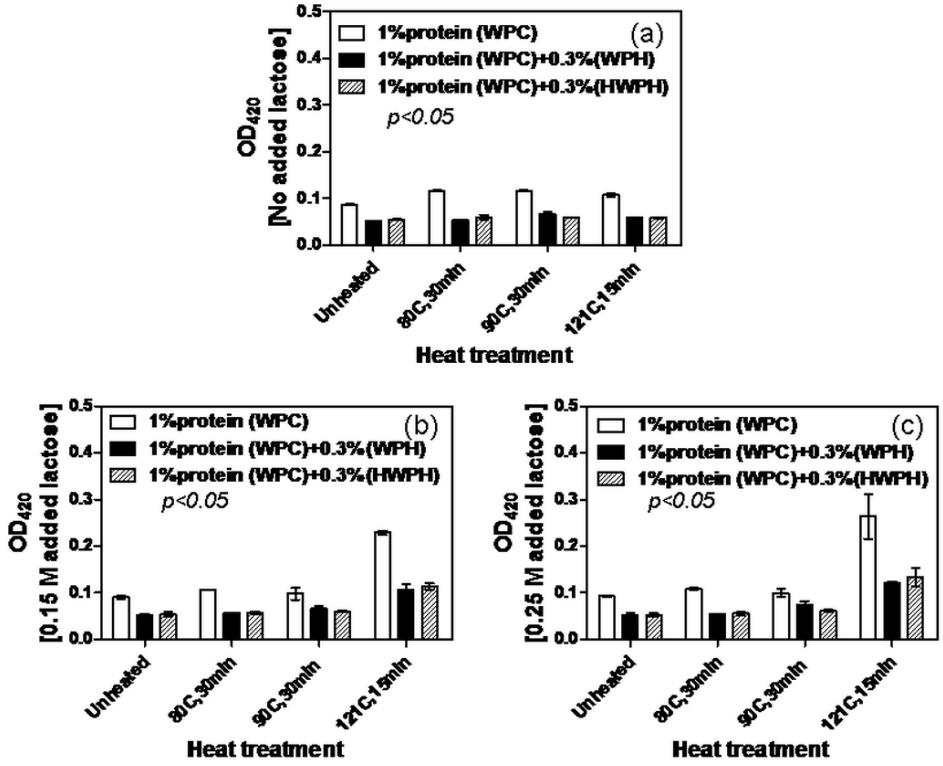

Fig. 4 Effect of whey protein hydrolysate (WPH) or heated whey protein hydrolysate (HWPH) addition on the reduction of brown pigment (measured as OD_{420}) of heated mixtures of whey protein concentrate (WPC) and hydrolysates (WPH or HWPH) in the presence of (a) 0 M; (b) 0.15 M and (c) 0.25 M added lactose. Bars represent standard deviation.

Commercial WPC used in this study contained carbohydrate high enough to cause a slight increase in OD_{420} after heat treatments (80 °C, 30 min; 90 °C, 30 min; and 121 °C, 15 min) compared to unheated WPC (P<0.05, **Fig. 4a**). The addition of lactose within a range of 0.15–0.25 M further enhanced brown pigment formation, particularly when the WPC-lactose mixtures were subjected to heat treatment at 121 °C for 15 min (P<0.05, **Fig. 4b, 4c**). The presence of reconstituted WPH and HWPH prior to heat treatment, however, reduced brown pigment formation even at high temperature (P<0.05).

The effect of tryptic hydrolysis on brown pigment formation and antioxidant capacity is illustrated in Fig. 5. Brown pigment formation could be significantly lowered by tryptic hydrolysis of WPC and HWPC (P<0.05; **Fig. 5a**). Moreover, both WPH and HWPH had higher $ORAC_{FL}$ antioxidant capacity than WPC and HWPC before hydrolysis (P<0.05; **Fig. 5b**). The $ORAC_{FL}$ antioxidant capacity of the reconstituted WPH and HWPH was increased from 0.2 μmol TE/mg protein in intact whey proteins to 0.5 μmol TE/mg protein in the hydrolysates (P<0.05).

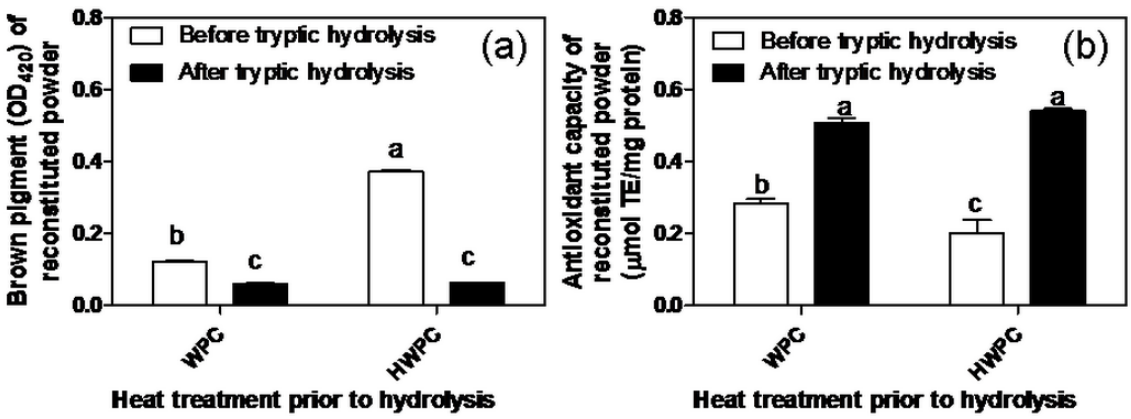

Fig. 5 Effect of tryptic hydrolysis on (a) brown pigment formation measured as OD_{420nm} and (b) $ORAC_{FL}$ antioxidant capacity. Bars represent standard deviation.

The decrease in brown pigment formation and the increase in $ORAC_{FL}$ antioxidant capacity may be involved in the survival of Caco-2 cells grown in media with added WPH and HWPH (**Fig. 6**). Two-way ANOVA suggested within the additional range of whey protein products below 2.5 mg protein/mL, the types of whey protein products (HWPC *vs.* hydrolysates) affected the survival of Caco-2 cells (P<0.05). Caco-2 cells grown in media containing reconstituted WPH and HWPH had similar survival to those grown in normal medium MEM (close to 100%). This suggested that the cytotoxicity of HWPC on Caco-2 cells (**Fig. 3b**) could be reduced after tryptic hydrolysis (**Fig. 5**).

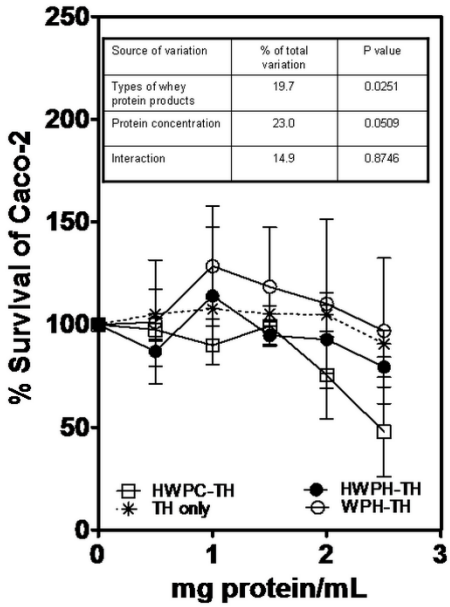

Fig. 6 Survival of Caco-2 grown in medium added with heated whey protein concentrate (HWPC), unheated whey protein hydrolysate (WPH), and heated whey protein hydrolysate (HWPH). The whey protein products were co-dried in trehalose (TH) using a spray-dryer. Dotted line represents the effect of TH excipient existing in whey protein products at the corresponding concentrations. Bars represent standard deviation.

The chemopreventive effect of WPH on the survival of FHs 74 Int and Caco-2 cells is illustrated in **Fig. 7**. FHs 74 Int and Caco-2 were subjected to HWPC at 3.5 mg protein/mL, a concentration close to the IC_{50} of HWPC on FHs 74 Int (3.38 mg protein/mL, Table 1), before and after the addition of WPH. Both FHs 74 Int and Caco-2 cells pretreated with WPH at 2.5 mg protein/mL prior to the addition of HWPC were able to survive better if they had taken up WPH prior to exposure to the cytotoxic level of MRPs in 3.5 mg protein/mL of HWPC. The cells exposed to HWPC prior to the uptake of WPH, however, had less than a 10% survival due to the cytotoxicity of MRPs from HWPC. This suggested a chemopreventive effect of antioxidative hydrolysate of WPH against MRPs cytotoxicity of HWPC on FHs 74 Int and Caco-2 cells.

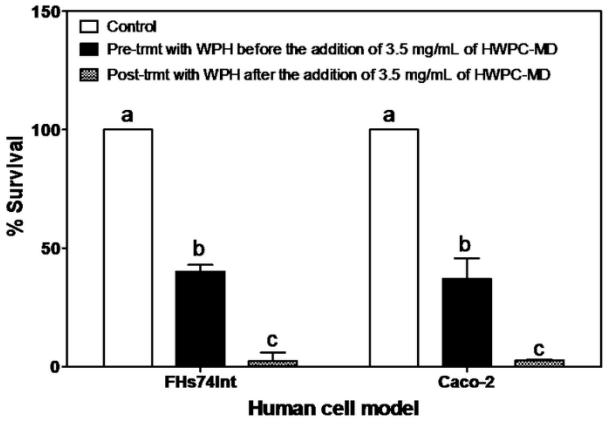

Fig. 7 Effect of the addition of reconstituted whey protein hydrolysate (WPH, 2.5 mg protein/mL) on the survival of normal human intestinal FHs 74 Int and Caco-2 cells against a cytotoxic level of the MRPs existing in heated whey protein concentrate (HWPC, 3.5 mg/mL). Bars represent standard deviation.

The cytotoxicity of MRPs formed by heating sugar-amino acid or sugar-protein mixtures is concentration-dependent. For example, MRPs in sugar-amino acid systems (xylose-lysine, glucose-lysine and fructose-lysine) could cause DNA damage in HepG2 cells even at 0.2 mg/mL of undialyzed sugar-amino acid mixture, although cytotoxicity was not observed (Yen & Liao, 2002). No significant toxicity of MRPs from glucose-lysine, fructose-lysine or sugar-casein mixtures was observed on Caco-2 cells when sugar-amino acid or sugar-casein mixtures were tested at a concentration of 1.0 mg/mL (Jing & Kitts, 2000; 2002; 2004). However, this study has extended the concentration of protein-carbohydrate mixture to the concentration of protein recommended in the diet (according to the RDA) in order to evaluate the possible risk of MRP cytotoxicity in thermally processed whey protein products.

The antioxidant activity determined by $ORAC_{FL}$ assay of around 0.5 μmol TE/mg protein of trypsin-hydrolyzed WPH and HWPH was relatively low compared to the purified fractions of protein hydrolysates from milk proteins (α-lactalbumin, α_{s2}-casein and κ-casein), which were within a range of 0.06 to 7.07 μmol TE/mg protein (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; López-Expósito, Quirós, Amigo, & Recio, 2007; Hogan, Zhang, Li, Wang, & Zhou, 2009). Nevertheless, the crude WPH and HWPH prepared in this study were able to prevent brown pigment formation at elevated temperature in HWPC suspension, and showed a chemopreventive effect against cytotoxic MRPs generated during heating of WPC in excess water. Overall, this study proposed the potential use of WPH and HWPH as antibrowning agents for thermally processed foods, and as chemopreventive antioxidants for oxidative stress caused by MRPs.

4. Conclusions

This study indicated that sugar-protein MRPs induced during heat treatment of WPC in excess water could have detrimental effects on brown pigment formation, and may cause a reduction of antioxidant capacity and an increase in cell cytotoxicity. The brown pigment formation and the low antioxidant capacity of HWPC were likely responsible for the reduction in the survival, particularly among normal human intestinal FHs 74 Int cells and epithelial colorectal carcinoma Caco-2 cells. Nevertheless, the formation of MRPs and cytotoxicity from MRPs induced by thermal processes could be reduced in the presence of WPH and HWPH after tryptic hydrolysis. Further investigations are needed to better understand the balance between the positive effect (antioxidant capacity) and negative effect (cell cytotoxicity) of MRPs on overall human health when MRPs are consumed in the diet.

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2.2 Mechanisms of Maillard browning reduction of whey protein hydrolysate powder in sterilized protein products

Abstract

This research investigated the mechanisms in the reduction of Maillard browning reactions of whey protein hydrolysate powder (WPH) prepared by hydrolysis of whey protein concentrate (WPC) with trypsin (E.C.3.4.21.4) at 37 ^{o}C for 60 min and spray-dried with trehalose when used in the mixed solutions containing protein 1.3 % (w/v) from WPC 1.0 % and WPH 0.3 %, added with lactose 0 – 0.25 mol L^{-1}, and sterilized at 121 ^{o}C for 15 min. The mechanisms of browning reduction of reconstituted WPH powder involved the increasing antioxidant activities of WPH, assessed by oxygen radical absorbance assay and $ABTS^{\bullet+}$ radical scavenging capacity assay of the short-chain peptides of less than 6.5 kDa. Besides, the thiol groups played an essential role in reducing the occurrence of brown melanoidin pigments, probably by directly involved in the Maillard reactions that generated fluorescent products or donating the hydrogen atom to the free radicals, and subsequently transforming to stable intermediates, thus suppressing the formation of brown pigments in full Maillard reactions of protein – lactose solutions subjected to sterilization.

1. Introduction

The Maillard reaction or glycation is essential in food processing and storage. It is also a reaction that occurs in living organisms. Although this reaction enhances the functional properties of foods such as water hydrophilicity, antioxidative activity, and functional properties of proteins and starches [1], the Maillard reaction end products (MRPs) may lead to the loss of nutrients and reducing the proportion of digested protein [2]. The formation of MRPs has both benefits and disadvantages in the food and beverage industry. On the positive side, MRPs are the source of color and taste. The advanced glycation end products (AGEs) and the melanoidins consist of eneaminol, enediol, and reductone, which had antioxidant capacity [3]. However, recent reports have shown toxicity of the MRPs to both mice and human cells [3, 4]. The accumulation of MRPs in biological systems has been linked to important pathogenesis in diabetes, vascular disease, Alzheimer's disease, and age-related diseases [3].

The initial Maillard reaction is due to the condensation of carbonyl groups of aldehydes and amino groups of proteins or amino acids, which leads to the formation of Schiff-base compounds and Amadori rearrangement reaction [5]. Although the ε-amino group of lysyl sidechain in proteins preferably reacts with carbonyl groups, other amino acids, such as methionine, cysteine, and tryptophan, can be involved in a Maillard reaction [2] due to the reactions of their side chains.

After the Amadori rearrangement, further Maillard reactions generated the highly reactive compounds or AGEs through condensation and fragmentation. The formation of AGEs is primarily related to redox reactions when Amadori products create intermediates such as dicarbonyls, oxoaldehydes, glyoxal, and methylglyoxal. Most AGEs are unstable and cause autooxidation between AGEs and cross-linking reactions [5].

The final stage of the Maillard reaction is due to the condensation of aldol and the condensation of aldehyde-amine, leading to the formation of a high MW N-containing brown pigment called melanoidins [5, 6]. The suppression of the occurrence of AGEs and MRPs can be achieved by inhibiting the oxidation mechanism using natural compounds, for example by using polyphenolic compounds [7, 8], amino acids [9], and peptides and proteins [4, 10, 11] to hinder the cross-linking pathway among reactive molecules in the Maillard pathway.

The production of peptides and bioactive protein hydrolysate and the sequence of amino acid residues in short-chain peptides has been extensively investigated [12, 13] for the inhibition of oxidation in cell models. However, little is known about the potential use of antioxidative food peptides and protein hydrolysates to reduce the Maillard reaction in sterilized foods. Moreover, the mechanism of inhibiting the brown pigment formation from the Maillard reaction is mostly not known. The objective of this study was to elucidate the mechanisms of Maillard browning inhibition of hydrolyzed whey protein (WPH) when used in sterilized commercial whey protein concentrate (WPC) solutions at different lactose levels. The insights from this research can be used in controlling the Maillard browning reaction in sterilized milk protein products. Trypsin (E.C.3.4.21.4) was chosen because it is a significant protease in mammals and specific in the hydrolyzing C-terminal to arginine and lysine [14], which will not interfere with cystine content.

2. Materials and methods

2.1 Materials

Commercial WPC was imported and repacked by a local distributor in Thailand. It contained 75.83% protein (w.b.), 6.5% moisture content (w.b.), 2.71% ash (w.b.), 1.05% fat (w.b.) and 13.91% carbohydrate (w.b.) [15]. Trypsin (E.C. 3.4.21.4; 10,000 BAEE U mg^{-1}), *o*-phthaldialdehyde (OPA), fluorescein (Na salt), and lactose monohydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, 1-anilino-8-naphthalenesulfonate (ANS), quinine, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Steinheim, Germany); 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 5,5′-dithio-bis-(2-nitrobenzoic acid)

(DTNB) and *N*-ethylmaleimide (NEM) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol 99% (DTT) was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate dihydrate, and sodium chloride were purchased from Ajax Finechem (Taren Point, NSW, Australia). Trehalose (kindly supplied by the East Asiatic Company, Bangkok, Thailand) was used as a carbohydrate excipient during spray-drying.

2.2 Characteristics of whey protein hydrolysates (WPH)

2.2.1 Effect of hydrolysis time on the molecular weight of proteins

WPC suspension (2% protein w/v) was prepared in distilled water and hydrolyzed by trypsin at 37 °C, pH 8.0, for 30, 60, and 90 min using protein to enzyme ratio of 100:1. The hydrolysis was inactivated by adjusting the pH to 2.0 using 5 mol L^{-1} HCl. The WPC and hydrolysates were characterized for molecular weight (MW) by glycine SDS-PAGE [16] using 12% separating gel and 4% stacking gel. An aliquot of the sample solution containing 7 µg protein, or 4 µL low-range MW standards (6,500 – 66,000 Da, Sigma-Aldrich, M3913) was loaded into each well. Electrophoresis was run at a constant current of 150 V for 45 min. Gel slabs were fixed and stained simultaneously using stain solution (10 % acetic acid, 40 % methanol, and 0.01 % Coomassie Brilliant Blue R-250 (Bio-Rad)) for 30 min, and then destained (10% methanol, 7.5% acetic acid, and 82.5% deionized water).

2.2.2 Preparation of whey protein hydrolysate powder

Commercial WPC suspension 7.5 % w/v, which contained 5.69 % protein w/v, was prepared in distilled water and hydrolyzed by trypsin using protein to enzyme ratio of 100:1 and hydrolysis time of 60 min at 37 °C using method described earlier [4]. Trypsin was inactivated by adjusting the pH to 2.0 using 5 mol L^{-1} HCl. The hydrolysate was mixed with trehalose (TH) excipient using a ratio of protein to the carbohydrate of 0.3:0.7. Trehalose was used to embed proteins and peptides in its amorphous matrix upon drying and storage due to the high glass transition temperature of TH around 115-117 °C [17]. The suspensions containing the total solid of 20 % w/v were fed at a rate of 4.0 ± 0.5 mL min^{-1}, and dried using an inlet air of 130 °C and outlet air of 100 °C at a flow rate of around 600 Nm^3 min^{-1} using a Mini Spray Dryer B-190 (Büchi, Flawil, Switzerland). The WPH-TH powder was packed in a sealed aluminum foil bag and stored at -20 °C before analysis.

2.2.3 Determination of amino groups

Amino group content was determined by *the o*-phthaldialdehyde (OPA) method [18]. The amount of reactive NH_2 groups was obtained by measuring the absorbance at 340 nm using a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland). All samples were determined in triplicate, using distilled water as a blank; values were reported as absorbance at 340 nm.

2.2.4 Determination of thiol groups

Thiol group contents in reconstituted commercial WPC and WPH-TH were determined using Ellman's reagent [19]. The absorbance was measured at 412 nm using a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland).). The thiol contents were calculated according to Eq. (1):

$$SH\ content\ (\mu\ mole\ g^{-1}\ protein) = \frac{73.54 \times Abs_{412} \times 6.04}{Sample\ concentration\ (mg\ mL^{-1})} \quad (1)$$

2.2.5 Oxygen radical absorbance capacity ($ORAC_{FL}$)

Reconstituted commercial WPC and WPH-TH (1.3% protein w/v) were prepared in 0.1 mol L^{-1} phosphate buffer, pH 8.0, and evaluated for oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) as determined by fluorescence assay [20, 21] in comparison with commercial WPC suspensions. The $ORAC_{FL}$ assays were carried out on a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) and reported as μmol Trolox equivalent per mg of protein (μmol TE mg^{-1} protein).

2.2.6 $ABTS^{\bullet+}$ radical scavenging capacity (Trolox equivalent antioxidant capacity, TEAC)

Suspensions of commercial WPC and WPH-TH (1.3% protein w/v) were prepared in 5 mmol L^{-1} phosphate-buffered saline (PBS), pH 7.4, and evaluated for Trolox equivalent antioxidant capacity (TEAC) [22]. The $ABTS^{\bullet+}$ solution was diluted with PBS to an absorbance of 0.700 at 734 nm as measured by a spectrophotometer (Spectronic 20+; Thermo Fisher Scientific, Waltham, MA, USA). Thirty μL of the sample was added to 3 mL of $ABTS^{\bullet+}$ solution, equilibrated at 30 °C for 4 min. Then, it was measured for absorbance at 734 nm using Trolox as an antioxidant standard. PBS was used as a blank. All determinations were carried out in triplicate. The absorbance at 734 nm was plotted as a function of antioxidant concentration. Each sample's antioxidant capacity was calculated as μmol Trolox equivalent per mg of protein (μmol TE mg^{-1} protein).

2.3 Antibrowning characteristics of reconstituted whey protein hydrolysate in sterilized mixed WPC-lactose suspensions

2.3.1 Effect of thiol-blocking agent and sterilization on Maillard reactions in WPH.

Five mL of mixed WPH-TH (1.3% protein w/v) and 0.25 mol L^{-1} added lactose was prepared in a 20 mL test tube with a cap and sterilized at 121 °C for 15 min in an autoclave in the absence or presence of thiol-blocking agent *N*-ethylmaleimide (NEM). Before sterilization, NEM was added to block SH groups in reconstituted WPH. The final concentrations of NEM were 0, 15, 20, and 25 mmol L^{-1}. After sterilizing and cooling to 80 °C in an autoclave, the sterilized WPH was cooled down to 27 °C (room temperature) using running water within 30 min. The samples were then determined for AGEs and brown pigment formation using the methods described below.

2.3.2 Effect of WPH-TH addition on Maillard reactions in sterilized whey protein – lactose mixed suspension

Commercial WPC suspension (1 – 1.3 % protein w/v) in 0.1 mol L^{-1} phosphate buffer, pH 8.0, was supplemented with appropriate concentration of lactose solution, with or without WPH-TH (0 – 0.3% protein w/v). The final concentration of proteins in suspension was 1.3% (w/v) and added lactose concentration of 0, 0.0625, 0.125, 0.188 and 0.25 mol L^{-1}. The mixed suspensions were sterilized at 121 °C for 15 min, cooled at room temperature and characterized for reactive NH_2 group contents after sterilization by the OPA method described above. The antibrowning activity was evaluated as AGE formation and brown pigment formation.

2.3.4 Determination of advanced glycation end products (AGEs)

The amount of advanced glycation end products (AGEs) was measured as % fluorescence intensity using an excitation wavelength of 380 nm and an emission wavelength of 465 nm, as measured by a Tecan multifunctional microplate reader (Infinite® M200 PRO; Männedorf, Switzerland). All samples were determined in triplicate. Quinine sulfate (1 µg mL^{-1} in 0.1 N H_2SO_4) was used as a reference. The formation of AGEs was reported as % fluorescence intensity compared to that of quinine sulfate as 100% [23].

2.3.5 Evaluation of brown pigment formation

The end-stage of Maillard reactions was detected as brown pigment formation by measuring the absorbance at 420 nm and correcting for turbidity by subtracting the absorbance at 620 nm [24] using a Tecan multifunction microplate reader (Infinite® M200 PRO; Männedorf, Switzerland).

2.4 Statistical analysis

Experiments were carried out in two independent trials of spray-dried WPH-TH preparation; each trial was analyzed in triplicate. The data were analyzed by analysis of variance (ANOVA) with significance at $P<0.05$. Tukey's test determined significant differences among mean values. All statistical analyses were performed using Graphpad Prizm 8.4.2 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1 Characteristics of whey protein hydrolysates

The molecular weight profiles of commercial whey protein concentrate (WPC) and hydrolyzed whey protein (WPH) obtained after hydrolysis by trypsin for 30, 60, and 90 min, compared to the suspension before hydrolysis (WPH 0) are shown in **Fig. 8**. SDS-PAGE showed that the major proteins in commercial WPC (β-lactoglobulin (β-Lg, MW 18 kDa) and α-lactalbumin (α-La, MW 14 kDa)) retained after 90 min incubation with trypsin. Commercial WPC before hydrolysis, however, contained additional proteins having MWs between 29 and 36 kDa, between 45 and 66 kDa, and above 66 kDa. These proteins were likely polymerized products of the indigenous milk proteins that existed in commercial WPC before tryptic hydrolysis.

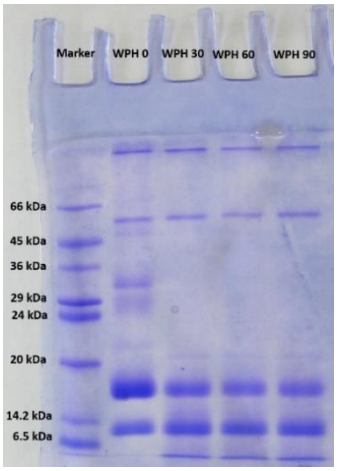

Fig. 8 Effect of hydrolysis time on the MW profiles of commercial WPC before hydrolysis (WPH 0) and after hydrolysis at 37 °C for 30, 60, and 90 min designated as WPH 30, WPH 60 and WPH 90, respectively.

Trypsin partially hydrolyzed some proteins in the commercial WPC (**Fig. 8**). However, β-Lg and α-La were hydrolyzed to some extent, observed as their less intense bands. Trypsin specifically hydrolyzes C-terminal to arginine and lysine [14]. The native whey proteins are quite resistant to tryptic hydrolysis and could be hydrolyzed by trypsin for less than 20% [25]. The complete degradation of α-La in WPCs by trypsin at 37 °C and pH 8 could take 120 min while β-Lg would take longer time for trypsin to hydrolyze [26]. Nonetheless, the presence of low-MW peptides of less than 6.5 kDa after hydrolysis for 30 – 90 min was observed in this study. The proteins having MWs within the ranges of 29 to 36 kDa and 45 to 66 kDa were entirely hydrolyzed after 30 min hydrolysis.

The characteristics of reconstituted commercial WPC and WPH-TH are shown in **Table 1.** The preparation of spray-dried WPH-TH increased NH_2 group content significantly but did not affect the thiol (SH) group. Approximately 1.5 fold increase in the NH_2 group content of WPH-TH determined by the OPA method suggested that the degree of hydrolysis was quite low, probably due to the compact globular structure of whey proteins not easily digested unless they were unfolded and exposed specific peptide bonds of arginine and lysine before hydrolysis as suggested by [25].

The increase in antioxidant capacities measured as $ORAC_{FL}$ and TEAC in WPH-TH, compared to that of commercial WPC, was probably due to greater exposure of reactive groups responsible for antioxidant activities in the hydrolysates, such as thiol (SH) group in cysteine residue. The small MWs peptides in WPH obtained after tryptic hydrolysis could also play significant roles in hydrogen atom transfer and electron transfer, especially if they contained aromatic ring side chain such as histidine, tryptophan, phenylalanine, tyrosine and proline. However, the tryptic hydrolysis for 60 min

investigated in this study was quite limited to minimize the exposure of aromatic amino acid that could generate bitter taste. The $ORAC_{FL}$ of commercial WPC was 0.20 μmol TE mg^{-1} protein before tryptic hydrolysis, which increased to 0.34 μmol TE mg^{-1} protein in WPH-TH (*P<0.05*). Likewise, the TEAC of WPH-TH was higher than that of the unhydrolyzed commercial WPC (*P<0.05*).

Table 1 Chemical characteristics of reconstituted whey protein products.

Types of whey protein powder	OPA NH_2 content reported as absorbance at 340 nm	Accessible SH content (μ mole g^{-1} protein)	Oxygen radical absorbance capacity ($ORAC_{FL}$) (μmol TE mg^{-1} protein)	Trolox equivalent antioxidant capacity (TEAC) (μmol TE mg^{-1} protein)
Commercial WPC	0.239^{b}±0.034	21.3^{a}±0.2	0.20^{b}±0.05	0.08^{b}±0.01
WPH-TH	0.361^{a}±0.007	20.8^{a}±0.5	0.34^{a}±0.00	0.12^{a}±0.00

Means ± SD in the same column from 2 independent trials, followed by different superscripts, are significantly different ($P < 0.05$).

3.2 Antibrowning characteristics of whey protein hydrolysates

The indigenous lactose of 0.007 mol L^{-1} in the 1.3% whey protein suspension was high enough to induce brown pigment formation when sterilization. The sterilized commercial WPC suspensions were light brown (**Fig. 9a**). However, the addition of WPH to WPC before sterilization lowered the brown color in sterilized WPC+WPH having similar protein and lactose contents (**Fig. 9b**).

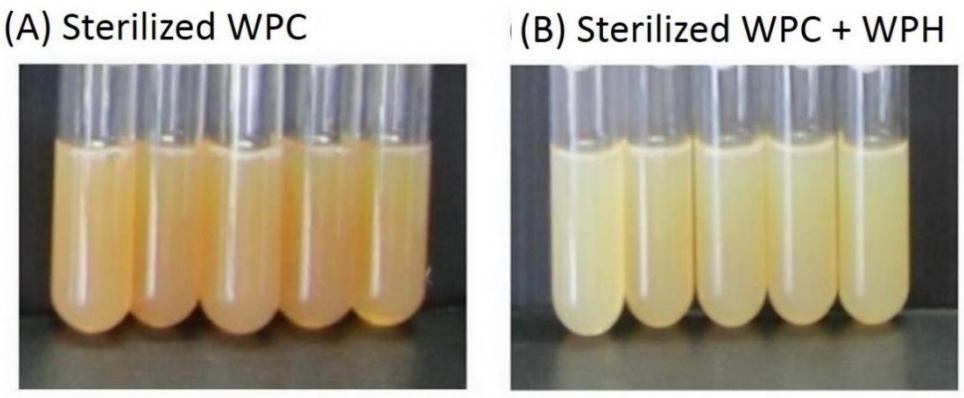

Fig. 9 Appearance of (A) sterilized commercial WPC containing 1.3% protein (w/v) and (B) sterilized commercial WPC (1.0 % protein (w/v) added with WPH (0.3% protein (w/v)). Final concentration of protein in suspension was 1.3 % (w/v) and indigenous lactose was 0.007 mol L^{-1}.

Fig. 10 shows that when lactose was added at the level of 0.25 mol L^{-1} (i.e., 8.55 % w/v), the addition of antioxidative WPH-TH in WPC suspensions before sterilization suppressed the formation of AGEs (P <0.05). At a low concentration of indigenous lactose in WPC (0.007 mol L^{-1}), the fluorescence intensity of sterilized WPC and WPC+WPH-TH was around 22% of quinine sulfate, and the influence of WPH was not apparent. However, lactose 0.25 mol L^{-1} drastically increased the formation of AGEs in sterilized WPC (P<0.05). The presence of antioxidative WPH-TH helped lower the formation of fluorescent AGEs in sterilized WPC+WPH-TH) when lactose concentration was increased, suggesting that WPH-TH could suppress further redox reactions of Amadori products that formed fluorescence compounds.

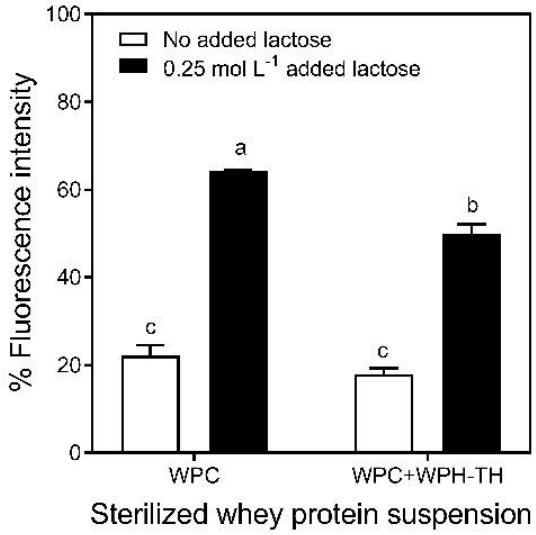

Fig. 10 Effect of WPH-TH addition in WPC suspensions on the formation of advanced glycation end products (measured as % fluorescence intensity) in sterilized protein suspensions. Total protein concentration was 1.3 % (w/v). Bars represent the standard deviation of 2 independent trials.

The SH-group roles in WPH-TH in antibrowning ability in the presence of 0.25 mol L^{-1} added lactose were further investigated. NEM blocked the SH groups in WPH-TH before sterilization. **Fig. 11a** shows that in the absence of NEM, fluorescence AGEs found in sterilized WPH-TH were around 57% of quinine sulfate (**Fig. 11a**). When NEM blocked SH group, sterilized WPH-TH had a lower formation of fluorescence AGEs (P<0.05) to 20%, suggesting that the SH group in reconstituted WPH-TH also participated in the formation of AGEs in addition to the well-known ε-NH_2 group of lysyl residue.

The formation of brown pigment in sterilized WPH-TH in the presence of 0.25 mol L^{-1} added lactose was not affected by SH-blocking agent NEM ($P \geq 0.05$), suggesting that the AGEs induced by SH group did not proceed to brown pigment formation. The SH group's involvement from cysteine residue was likely to form fluorescence intermediates from Amadori products rather than the condensation to polymerized brown-colored N-containing compound melanoidins in the final stage.

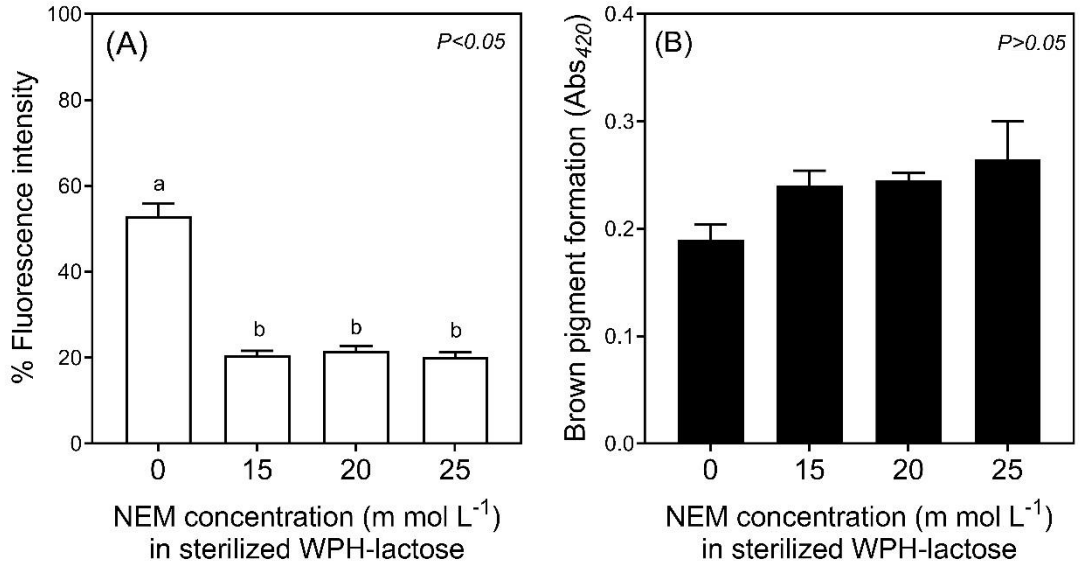

Fig. 11 Effect of thiol-blocking agent N*-ethylmaleimide (NEM) on the formation of (A) advanced glycation end products (measured as % fluorescence intensity) and (B) brown pigment formation (measured as absorbance at 420 nm) of sterilized WPH-lactose mixed suspensions containing 1.3% w/v protein and 0.25 mol* L^{-1} *added lactose. Bars represent the standard deviation of 2 independent trials.*

Cysteine residue could donate H atom and form stable complexes [9] is, in part, responsible for the antibrowning ability of WPH-TH and reduced brown color shown in **Fig. 9**. The SH group of cysteine not only inhibited the formation of AGEs by donating H atom to radicals in both early and advanced stages of Maillard reactions but could also react directly with dicarbonyl compounds to give stable *S*-carboxymethylcysteine [27], thus reducing the full length of Maillard brown color shown in **Fig. 9b**.

The influences of lactose concentration on chemical characteristics of sterilized WPC, WPC+WPH-TH, and WPH-TH suspensions (1.3% protein) are shown in **Fig. 12**. In the absence of added lactose, sterilized WPC+WPH-TH and WPH-TH suspensions (containing 0.007 mol L^{-1} indigenous lactose) had slightly higher NH_2 group content than did the sterilized WPC suspensions (**Fig. 12a**; (P<0.05)) due to the presence of peptides and free NH_2 groups in the hydrolysates. Despite the higher NH_2 group, the presence of WPH-TH before sterilization helped lower the formation of AGEs (**Fig. 12b**) and a much lower brown pigment formation (**Fig. 12c**) in sterilized WPC+WPH and WPH. It should be noted that although WPH investigated in this study contained intact β-Lg and α-La with a slight amount of less than 6.5 kDa peptides (**Fig. 8**) and similar contents of SH group (Table 1), the antioxidative WPH could help to reduce the formation of brown pigment.

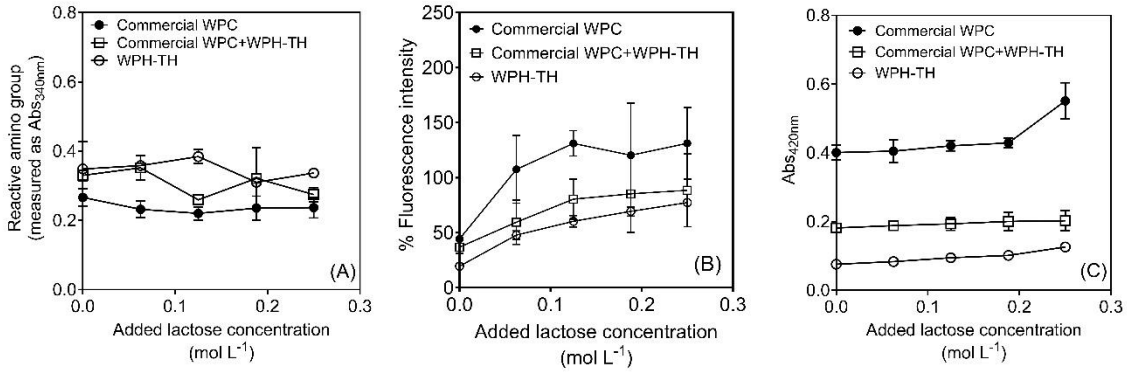

Fig. 12 Effect of added lactose concentration on (A) reactive amino group content, (B) advanced glycation end products measured as % fluorescence intensity, and (C) brown pigment formation measured as absorbance at 420 nm in sterilized protein-lactose suspensions containing 1.3% protein (w/v). Bars represent the standard deviation of 2 independent trials.

Raising the lactose concentration to 0.25 mol L^{-1} enhanced the formation of AGEs (**Fig. 12b**) and brown pigment (**Fig. 12c**) in sterilized WPC. However, the presence of WPH suppressed brown pigment formation in sterilized suspensions, observed as low value of absorbance at 420 nm of sterilized WPC+WPH and WPH suspensions. In the absence of WPH, adding lactose at the level of 0.0625 mol L^{-1} increased the formation of AGEs (**Fig. 12b**) in sterilized WPC. Further increase in lactose concentration did not significantly change % fluorescence intensity of sterilized WPC ($P \geq 0.05$). The brown pigment formation in sterilized WPC-lactose suspensions remained similar up to 0.20 mol L^{-1} added lactose, then dramatically increased when lactose concentration was added at 0.25 mol L^{-1} while % fluorescence intensity remained plateau (**Fig. 12c**).

The effect of lactose concentration on the formation of AGEs in sterilized WPC+WPH and WPH was not as drastic as found in sterilized WPC, probably due to the effectiveness antioxidative WPH in suppressing the formation of fluorescence AGEs at high lactose concentration. The present study suggested that the antibrowning ability of antioxidative WPH was *via* hydrogen atom transfer reaction (measured by $ORAC_{FL}$ assay) and electron transfer reaction (measured by TEAC assay).

This study provides information on the possibility of tryptic protein hydrolysate powder prepared in the presence of trehalose (TH) excipient to suppress the formation of AGEs and further brown pigment formation of N-containing melanoidins in sterilized whey protein-lactose mixed suspension. TH itself is not a reducing sugar. It has no antibrowning or antioxidant activities (result not shown) and was added to immobilize antioxidative whey peptides in the powder's glassy matrix. Storage stability of WPC containing antibrowning WPH in different carbohydrate excipients is underway.

4. Conclusion

This study demonstrated that reconstituted antioxidative WPH obtained from tryptic hydrolysis for 60 min, which partially hydrolyzed proteins, could reduce Maillard browning reactions induced during sterilization of whey proteins and lactose at 121 °C for 15 min. The antibrowning mechanisms involved the inhibition of brown pigment formation by lowering the formation of AGEs via antioxidative activities of reconstituted WPH by short-chain peptides of less than 6.5 kDa not present in the commercial WPC. The insights from this research could help control the formation of brown color pigments in thermally processed protein drinks.

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บทที่ 3 การผลิตโปรตีนไฮโดรไลเสทต้านอนุมูลอิสระจากโปรตีนถั่วเขียวที่ถูกตรึงในสารกระสัยคาร์โบไฮเดรตชนิดต่างๆ ระหว่างการทำแห้งแบบพ่นฝอยเพื่อใช้เป็นสารพาสารต้านมะเร็งกรดเอเซียติกจากใบบัวบก

3.1 Potential use of antioxidative mungbean protein hydrolysate as an anticancer asiatic acid carrier

ABSTRACT

This study investigated the potential use of mungbean (*Vigna radiata* (L.) Wilczek) protein hydrolysate (MPH) prepared from tryptic hydrolysis as an antioxidative hydrolysate and as a carrier for anticancer asiatic acid (AA). The antioxidant capacity of MPH was 0.67 and 0.46 μmol Trolox equivalent (TE)/mg protein, as measured by oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) and Trolox equivalent antioxidant capacity (TEAC) assays, respectively. Freeze-drying in lactose excipient reduced the antioxidant capacity of MPH to 0.48 μmol TE/mg protein in $ORAC_{FL}$ assay ($P<0.05$) but did not alter antioxidant capacity determined by TEAC assay ($P\geq0.05$). The genotoxicity of H_2O_2 (50 μM, 30 min) on hepatoblastoma HepG2 could be alleviated after HepG2 cells had taken up MPH after H_2O_2 exposure ($P<0.05$). Moreover, the inhibition concentration (IC_{50}) of AA in HepG2 was lowered from 58.5 μg mL^{-1} of AA alone to 38.5 μg mL^{-1} when AA was freeze-dried with MPH in lactose excipient ($P<0.05$). This study suggested that the efficacy of anticancer pentacyclic triterpene AA against hepatoblastoma HepG2 could be increased by co-drying with antioxidative mungbean protein hydrolysate in lactose excipient.

1. Introduction

After hydrolysis, the biofunctional properties of protein can be improved. The resulting peptides usually have biological activities other than being energy or essential amino acid sources. Their bioactivities include antihypertensive, hypocholesterolemic, antiobesity, antioxidative, anticancer and immunomodulatory activities. Most biofunctional properties rely on amino acid sequences in the polypeptides, the type of enzyme used, and the peptide recovery methods. Various plant and animal protein hydrolysates are used as sources of antioxidative peptides (Shahidi & Zhang, 2008). Recently, plant food protein hydrolysates such as soy proteins have received much attention as sources of bioactive peptides that may prevent age-related chronic diseases (Wang & de Mejia, 2005). Mungbean (*Vigna radiata* (L.) Wilczek), commonly known as "green gram," contains 19.1–28.3% protein on dry basis, depending on varieties and sources (Duke, 1981). It is rich in lysine; but the sulfur-containing amino acids, e.g. methionine and cysteine, are deficient (Coffmann & Garcia, 1977).

Mungbean is an important source of protein for the human food supply in Asia. Mungbean protein is also a by-product from vermicelli production and sold as animal feed in some countries. The major protein in mungbean seeds is vicilin (8S), which accounts for 89% of globulins, followed by 7.6% of 11S, and 3.4% of basic 7S globulin (Tecson-Mendoza, Adachi, Bernardo, & Utsumi, 2001). Only the basic 7S and 11S possess disulfide bonds. Mungbean protein isolate can perform many desirable functions in processed foods, such as foaming, emulsification and water absorption (El-Adawy, 2000).

Asiatic acid (AA) is a pentacyclic triterpene found in *Centella asiatica* (L.) Urban, a medicinal plant cultivated as a vegetable in Asia and known as "Indian pennywort," or *gotu kola* in Malaysia and Indonesia, or *bua-bok* in Thailand. Extracts of *Centella asiatica* have been demonstrated to aid wound healing (Shukla, Rasik, Jain, Shankar, Kulshrestha, & Dhawan, 1999), act as antimicrobial or antiviral agents (Cowan, 1999; Yoosook, Bunyapraphatsara, Boonyakiat, & Kantasuk, 2000), and improve cognitive behavior (Gupta, Veerendra Kumar, & Srivastava, 2003). *Centella asiatica* also exhibits anti-inflammatory action by modulating the production of nitric oxide (Punturee, Wild, & Vinitketkumneun, 2004). In addition, it also shows potential as a chemopreventive agent for cancer (Babu, Kuttan, & Padikkala, 1995; Bunpo et al., 2004; Hsu, Kuo, Lin, & Lin, 2005; Yoshida et al. 2005). In human breast cancer cells, AA induced apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways (Hsu et al., 2005).

The low solubility (30.6 μM) of AA in phosphate buffered saline (PBS) is reported. This is because of the low hydrophile-lipophile balance (HLB), which is around 5.5, due to AA's molecular structure, which is close to that of bile acid (Rafat et al., 2008). AA contains both hydrophobic and hydrophilic moieties. Such a molecular structure promotes the association or micellization of AA in phosphate buffered saline (PBS); the critical micelle concentration (CMC) in PBS is around 15 μM. Such low solubility of AA in aqueous phase and the tendency to form micelle may limit the efficacy of AA in cell penetration. Nevertheless, the CMC of AA can be increased to 52–54 μM in the presence of dimethyl sulfoxide (DMSO); thus lowered the inhibition concentration of AA in animal cell models as reported by Rafat et al. (2008).

This study hypothesized that the complexation of AA with amphiphilic molecules like food peptides, which have higher solubility in aqueous phase than does AA, could enhance the efficacy of AA in a cancer cell model. Food proteins have been used as encapsulating agents and as the regulators for the release of the bioactive compounds during digestion (Augustin & Hemar, 2009). They have been granted GRAS (generally regarded as safe) status, with low side effects compared with synthetic polymers (Ikada & Tsuji, 2000; Augustin & Hemar, 2009). Although DMSO has been used for topical

application of pharmaceuticals, it has a distinct taste, as oyster- or garlic-like, and may not be suitable for food use.

Proteins from pulses such as peas, chickpeas, lentils and beans have been shown to have many chemopreventive and therapeutic acitivities for nutraceutical applications (Boye, Zare, & Pletch, 2010). We have attempted to use mungbean protein hydrolysate as a carrier for AA based on the excellent emulsifying characteristics of mungbean protein concentrate (Naprom & Hongsprabhas, 2007). It is most likely that the amphiphilic AA and mungbean protein hydrolysates could complex via non-covalent forces. The assembled mungbean protein hydrolysate-AA was further freeze-dried in different carbohydrate excipients: namely lactose, maltodextrin, and a mixture of the two. The solid form of these therapeutic substances could yield advantages in practical handling.

The objectives of this study were thus to investigate the potential uses of antioxidative mungbean protein hydrolysate as a carrier for AA. The insights into the interplay between the amphiphilic core material (AA), the peptide carrier (mungbean protein hydrolysate), and carbohydrate excipients may help in understanding the consequences of freeze-drying and the behavior of freeze-dried multi-phase delivery systems.

2. Material and methods

2.1. Materials

De-hulled mungbeans (Raitip, Bangkok, Thailand) were purchased from a local supermarket. Trypsin (EC 3.4.21.4; 10,000 BAEE Umg^{-1}), asiatic acid (AA), and fluorescein (Na salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Trolox (6–hydroxy-2,5,7,8-tetramethylchroman–2–carboxylic acid), potassium persulfate and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Fluka (Buchs, Switzerland). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate dehydrate, and sodium chloride were purchased from Ajax Finechem Pty Ltd. (Seven Hills, Australia). Neo-Maldex® cassava maltodextrin, with dextrose equivalent (DE) of 11.7 as determined by the method described by Lane & Eynon (1923), was purchased from Neotech Food Co., Bangkok, Thailand. Lactose monohydrate (Sigma Chemical Co., St. Louis, MO) and maltodextrin were used as carbohydrate excipients during freeze-drying.

2.2. Preparation of mungbean protein concentrates (MPC) and mungbean protein hydrolysate (MPH)

De-hulled mungbean grain was milled in a Mikro-Pulverizer (Hosokawa Micron Ltd., Osaka, Japan) to prepare mungbean flour. The flour suspension (6.67% w/v total solids) was dispersed in distilled water; the pH was adjusted to 8 using 2 M NaOH to extract proteins. The flour suspension was continuously stirred for 1 h at 25 °C and centrifuged at 14000 *g* (Sorvall RC-5C Plus; Sorvall/DuPont, Newtown, CT) at 15 °C for 30 min. Spent flour was discarded. The supernatant was heated at 80 °C for 30 min to inactivate trypsin inhibitor, and then cooled to room temperature (Naprom & Hongsprabhas, 2007). Five mM $NaHSO_3$ and 5 mM $CaCl_2$ were added, and the pH was adjusted to 4.5 using 2 M HCl. The suspension was kept at 4 °C for 2 h, and then centrifuged at 9000 *g* at 4 °C for 15 min. The supernatant was discarded, and mungbean protein concentrate (MPC) was kept at –20 °C before use.

Mungbean protein hydrolysate (MPH) was prepared by re-suspending the thawed MPC in distilled water to obtain 1.4 % protein w/v, adjusting the pH to 8.0 using 2 M NaOH and hydrolyzing by trypsin at 40 °C for 60 min using a mungbean protein to enzyme ratio of 31:1. The hydrolysate was mixed with AA (3.6% in 99% EtOH), the core material, in different carbohydrate excipients: namely lactose (L); lactose-cassava maltodextrin at a ratio of 1:1 (LM); and cassava maltodextrin (M), using a protein to AA to carbohydrate ratio of 1:0.06:7.5. The mixtures (10.9% w/v total solids) were frozen in liquid nitrogen and freeze-dried in a Heto FD 2.5 freeze-dryer (Heto Lab Equipment, Allerød, Denmark) to lower the moisture content to less than 10%. Then the mixtures were ground, passed through a 150 μm sieve, sealed in aluminum foil bags, and stored at –20 °C before use. Before analyses, the powder was reconstituted by dispersing in distilled water unless stated otherwise.

2.2.1. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (tricine-SDS-PAGE)

Freshly prepared MPH and reconstituted MPH, in the absence or presence of AA, were characterized for molecular weight (MW) profiles by tricine SDS-PAGE (Schägger & von Jagow, 1987) separating gel. The continuous buffer contained 3 mM Tris-HCl (pH 8.45) and 0.3% SDS in stacking gel. The anode running buffer (outside) contained 0.02 M Tris-HCl (pH 8.9); while the cathode running buffer (inside) contained 0.1 M Tris, 0.1 M tricine and 0.1% SDS. MPH-AA samples, before and after drying, were added to dissociating buffer (0.5 M Tris-HCl [pH 6.8], 5.5% SDS, 7% glycerol, 0.01% Coomassie Brilliant Blue R-250, with or without 2% dithiothreitol [DTT]). Each solution was heated at 100 °C for 4 min, cooled, and centrifuged at 2125 g (Labnet Spectrafuge 16 M, Labnet International, Woodbridge, NJ) for 5 min to remove insoluble material. An aliquot of the sample solution containing 0.02 mg protein or 20 μL low-range MW standards was loaded into each well.

Electrophoresis was run at a constant current of 35 mA for 4 h. Gel slabs were fixed and stained simultaneously using Bio-Rad Coomassie Blue R-250 stain solution (10% acetic acid, 0.025% Coomassie Blue R-250) for 2 h, and then de-stained by 10% acetic acid.

2.2.2. Turbidity

Freshly prepared MPH (1.27% protein w/v) was diluted with distilled water to obtain 0.13% protein w/v. AA was added and mixed thoroughly; then the mixture was diluted with distilled water to obtain final concentrations of 0.013 protein % w/v and 0.008% AA w/v. Optical density (OD) measurement was performed at 400 nm using a T-60 UV-visible spectrophotometer (PG Instruments, Wibtoft, Leicester, UK). The turbidity, which reflected the degree of aggregation, was calculated (Pearce & Kinsella, 1978) as:

$$Turbidity = \frac{OD_{400}}{Pathlength\ (cm)} \times 2.303$$

2.2.3. Antioxidant capacity assays

Freshly prepared MPH and reconstituted MPH, in the absence or presence of AA, were characterized for antioxidant capacity using: Trolox equivalent antioxidant capacity (TEAC) assays (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999; Sun, Hayakawa, Ogawa, & Izumori, 2007); oxygen radical absorbing capacity–fluorescein ($ORAC_{FL}$) assay (Prior et al., 2003; Dávalos, Gomez-Cordoves, & Bartolome, 2004); and alkaline comet assay to evaluate genotoxicity caused by H_2O_2 oxidative stress (Ostling & Johnson, 1984), as described below.

2.2.3.1. Trolox equivalent antioxidant capacity (TEAC) assays

Briefly, $ABTS^{\bullet+}$ radical solution was prepared by mixing 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in the dark for 16 h at room temperature, and diluted before use with PBS at pH 7.4 to achieve an absorbance of 0.700 ± 0.020 at 734 nm. Thirty μL of MPC or MPH (1.27% protein w/v) were mixed with 3 mL of $ABTS^{\bullet+}$ radical solution and incubated at 30 °C for 4 min. The absorbance at 734 nm was recorded using a spectrophotometer (Spectronic 20+, Thermo Fisher Scientific, Madison, WI). The calibration curve was prepared using concentrations of Trolox ranging from 0.1 to 2 mM. TEAC values were calculated by plotting the absorbance of decolorization of $ABTS^{\bullet+}$ radical solution vs. the concentration of Trolox. The TEAC of samples was expressed as μmol Trolox equivalent in 1 mg of protein in MPC or MPH (μmol TE/mg protein) powders.

2.2.3.2. Oxygen radical absorbance capacity–fluorescein ($ORAC_{FL}$) assay

The $ORAC_{FL}$ assay was performed on a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany). The reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4); the final reaction mixture was 200 μL. Antioxidant (20 μL; Trolox 2–9 μM or sample 1–3 mg/mL) and fluorescein (120 μL; 700 nM, final concentration) were loaded into a black, 96-well microplate reader which was programmed to record the fluorescence of fluorescein on every cycle. During cycle 4, a pump was programmed to inject 60 μL of AAPH into the respective wells to give a final AAPH concentration of 12 mM. The plate contents were mixed by shaking for 8 s. The temperature of the incubator was set to 37 °C, and a fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was used.
The final $ORAC_{FL}$ values were calculated using a linear equation between the Trolox standards or sample concentration and net areas under the fluorescein decay curves. The area under curve (AUC) was calculated as:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 +, ..., + f_i/f_4) \times CT$$

where f_4 was the initial fluorescence reading at cycle 4, f_i was the fluorescence reading at cycle *i*, and CT was the cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of a sample. $ORAC_{FL}$ values of samples were then reported as μmol Trolox equivalent per mg of protein in MPC or MPH (μmol TE/mg protein) powders.

2.3. In vitro antioxidant capacity of mungbean protein hydrolysate in lactose excipient in hepatoblastoma HepG2

Human hepatoblastoma HepG2 (ATTC HB-8065) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MA). HepG2 were plated in a 24-well plate at an initial density of 2×10^4 cells/well with Eagle's minimum essential medium (MEM; Gibco®, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), in accordance with ATCC recommendations. Media were filtered through a 0.2 μm polystyrene filter (Corning, Corning, NY). The cells were grown at 37 °C under 5.0% CO_2 atmosphere.

Control treatment: 48 h after initial seeding, the medium was replaced with new medium and HepG2 cells were grown for another 48 h.

H_2O_2 treatment: 48 h after seeding, the cells were exposed to 50 μM H_2O_2 for 30 min; then the medium was replaced with new medium and the cells were grown for 48 h.

Simultaneous treatment *of H_2O_2 and MPH*: 48 h after seeding, the cells were exposed to 50 μM H_2O_2 and 8 mg/mL MPH at the same time for 30 min; then the medium was replaced and the cells were grown for 48 h.

Post-treatment with MPH: 48 h after seeding, the cells were exposed to 50 μM H_2O_2 for 30 min; then the medium was replaced with new medium containing 8 mg/mL MPH and the cells were grown for 48 h. Genotoxicity of H_2O_2 on HepG2 in each treatment, compared to the control treatment, was assessed by alkaline comet assay (Ostling & Johnson, 1984).

The cells were trypsinized and centrifuged to wash out growth medium. Then the cell pellets (10^5 cells in 0.5 mL PBS) were suspended in 0.5% low-melting-point agarose, covered with a cover slip, and placed over ice cubes to set the gel. After the gel was set, the cover slip was removed and the cells were lysed for 1 h with cold lysis buffer (pH 10) containing 2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO. Slides were then incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 40 min at 4 °C, followed by electrophoretic separation in Tris-acetate-EDTA (TAE) buffer at 25 V, 300 mA, for 1 h. The slides were then rinsed with ethidium bromide for 5 min before washing with cold distilled water. Images from five different observation fields were taken by an Olympus BX61 epifluorescence microscope equipped with a digital camera (Olympus America, Melville, NY).

Cells with comet images were counted based on total cells between 900–4,700 cells, depending on the treatment. Comet tail lengths (comprising nuclear region and tail) for each cell were manually classified into five comet classes: ***Class 0*** (undamaged, no tail); ***Class 1*** (tail up to 1.5 times the comet nucleus diameter); ***Class 2*** (tail 1.5–2.0 times the comet nucleus diameter); ***Class 3*** (tail 2.0–2.5 times the comet nucleus diameter); and ***Class 4*** (maximally damaged, tail >2.5 times the comet nucleus diameter). The cell number counted in each class was divided by the total cell count to estimate the % population prone to genotoxicity of H_2O_2, both in the absence or presence of MPH.

2.4. Cytotoxicity of asiatic acid (AA) and mungbean protein hydrolysate-AA complex (MPH-AA) on hepatoblastoma (HepG2)

The 500 μg/mL AA stock solution was prepared in 1% DMSO, then diluted with media to obtain media having 0–75 μg/mL AA. HepG2 cells were plated in a 24-well plate at an initial density of 2 x 10^4 cells/well with MEM supplemented with 10% FBS. Forty-eight h after seeding, the media were replaced with new media supplemented with predetermined doses of AA and MPH-AA, co-dried in lactose or lactose-maltodextrin excipients. The cells were grown for another 48 h to determine the 50% inhibition concentration (IC_{50}) of AA and MPH-AA in HepG2. Cell viability was evaluated by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) reduction assay (Invitrogen, Carlsbad, CA).

2.5. Statistical analysis

The mixtures and their freeze-dried products were prepared in two separate trials. The data were analyzed by analysis of variance (ANOVA) with significance at $P<0.05$. Significant differences among mean values were determined by Duncan's multiple range test. All statistical analyses were performed using SPSS software Version 12.

3. Results and discussion

The presence of 0.008% of AA in MPH suspension promoted aggregation, measured as increasing turbidity at 400 nm in the presence of AA (**Table 2**). Although AA was solubilized in EtOH prior to the mixing of AA with MPH, EtOH-induced aggregation of MPH was negligible (result not shown). AA is an amphiphilic surfactant, which has three hydroxyl groups and one carboxylic group in the hydrophilic region, and pentacyclic triterpene with methyl groups in the hydrophobic part (Rafat et al., 2008). Therefore, AA can increase turbidity due to the induction of peptide-peptide or peptide-AA associations. The structure of AA is close to that of bile acid. This, in turn, could form ionic interactions between the positively charged ε-amino group of lysine and the negatively charged carboxylic group in AA. Hydrophobic interactions and van der Waal's forces could also be involved in these associations (Roda et al., 1982).

Table 2 Effect of asiatic acid (AA) and types of carbohydrate on the aggregation, determined as turbidity, and antioxidant capacity of freshly prepared mungbean protein hydrolysate (MPH) suspensions. The suspensions contained 0.013% protein and 0.008% AA.

Type of carbohydrate (protein:carbohydrate ratio = 1:7.5)	Turbidity at 400 nm		$ORAC_{FL}$ antioxidant capacity of freshly prepared MPH (μmol TE / mg protein)[ns]	
	No AA	With AA	No AA	With AA
None (MPH alone)	1.76^{cd}	2.87^{a}	0.567	0.479
Lactose	1.53^{d}	2.68^{ab}	0.457	0.497
Lactose:maltodextrin (1:1)	1.58^{d}	2.28^{bc}	0.492	0.426
Maltodextrin	1.58^{d}	1.98^{cd}	0.503	0.556

Means followed by different superscripts are significantly different ($P<0.05$).

[ns] Indicates no significant difference ($P\geq0.05$).

In the absence of AA, the carbohydrate excipients did not affect aggregation although they were present at a protein to carbohydrate ratio of 1:7.5 ($P\geq0.05$). The presence of high MW carbohydrates, however, lowered the turbidity of MPH in the presence of AA (**Table 3**). Increasing the MW of carbohydrate excipient resulted in a lower degree of aggregation ($P<0.05$). This was likely due to the higher viscosity of the solution in the presence of maltodextrin, which delayed aggregation.

The $ORAC_{FL}$ antioxidant capacity of freshly prepared MPH was around 0.57 μmol TE/mg protein. The presence of AA or carbohydrate excipients did not significantly affect the $ORAC_{FL}$ antioxidant capacity of freshly prepared MPH, although the AA could induce the aggregation of MPH.

Most mungbean proteins were hydrolyzed to MWs of less than 10.7 kDa. The presence of AA and carbohydrates did not affect the MWs of the protein hydrolysates (**Fig. 13a**), both in the absence or presence of DTT, although they induced aggregation which was observed as increased turbidity (**Table 3**). This suggests that the aggregation of MPH in the presence of AA involves weak forces that could be dissociated in SDS-PAGE buffering solutions.

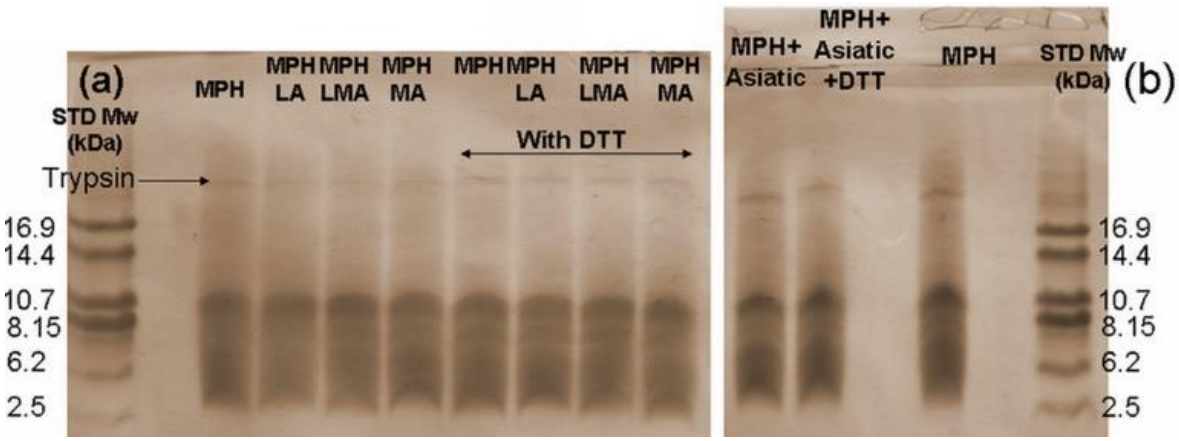

*Fig. 13 Effect of carbohydrate excipients on the MWs of **(a)** freshly prepared mungbean protein hydrolysate (MPH) and **(b)** reconstituted MPH co-dried with lactose excipient. L = lactose; M = maltodextrin; A = asiatic acid; DTT = dithiothreitol.*

The result of SDS-PAGE indicates no alteration in the MWs of the hydrolysates after reconstitution (**Fig. 13b)**. Most of the proteins in hydrolysates had MWs of around 10.7 kDa and below, both in the absence and presence of DTT. This suggests that co-drying of MPH and AA in carbohydrate excipient did not alter the MWs of the peptides. However, freeze-drying lowered the antioxidant capacity of both MPC and MPH in lactose excipient compared to the freshly prepared ones as determined by $ORAC_{FL}$ assay (**Table 3**).

Table 3 Effect of freeze-drying in lactose excipient on antioxidant capacity of mungbean protein (MPC) and mungbean protein hydrolysate (MPH).

Type of protein	Antioxidant capacity (μmol TE / mg protein) of MPH			
	$ORAC_{FL}$ assay		TEAC assay	
	Freshly prepared	Reconstituted	Freshly prepared	Reconstituted
MPC	0.077^{c}	0.055^{d}	0.150^{B}	0.245^{AB}
MPH	0.670^{a}	0.484^{b}	0.455^{A}	0.465^{A}

Means in the same assay followed by different superscripts are significantly different (P<0.05).

Table 3 indicates that tryptic hydrolysis significantly increased the antioxidant capacity of MPH – by almost 9 and 3 times, as measured by $ORAC_{FL}$ and TEAC assays, respectively, compared to that of unhydrolyzed MPC (P<0.05). Hydrolysis not only shortened the polypeptides, but also exposed the potent reactive groups of amino acid side chains in antioxidation, i.e. aromatic and nucleophilic side chains, as well as the thiol group of cysteine buried within the core of the protein structure (Shahidi & Zhong, 2008).

Different carbohydrate excipients also altered the antioxidant capacity of reconstituted MPH (**Fig. 14**) although they did not significantly affect the antioxidant capacity of MPH-excipient mixture prior to dehydration (**Table 3**). Co-drying of MPH and MPH-AA with maltodextrin, which had the highest viscosity compared to lactose and mixed lactose/maltodextrin, resulted in the lowest $ORAC_{FL}$ antioxidant capacity of reconstituted MPH. However, there was no detectable change in TEAC antioxidant capacity before or after freeze-drying (**Table 3** and **Fig. 14**, respectively), regardless of the type of carbohydrate excipient ($P \geq 0.05$). The mechanisms involved in the reduction of $ORAC_{FL}$ antioxidative capacity of MPH induced by different carbohydrate excipients before and after dehydration is currently investigated.

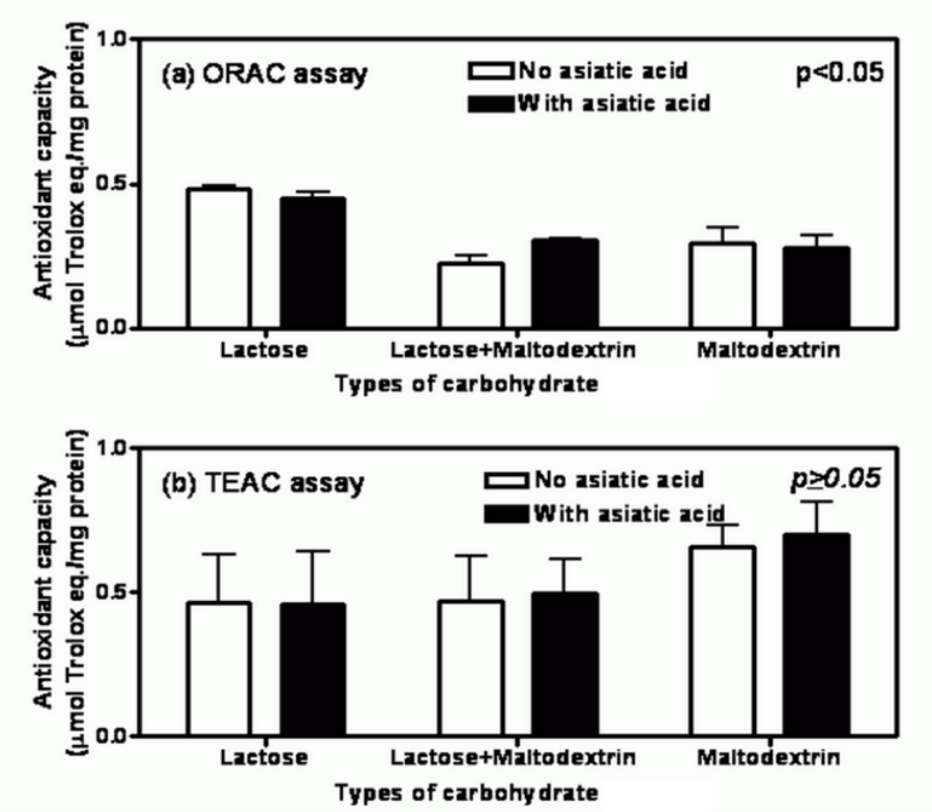

Fig. 14 Effect of carbohydrate excipients on antioxidant activity of reconstituted mungbean protein hydrolysate (MPH) in the absence and presence of asiatic acid (AA).

Aggregation of peptides in MPH may be subjected to frozen concentration in carbohydrate excipients prior to sublimation during the freeze-drying process. Different carbohydrate excipients were used in this study. Although they were most likely in glassy states after proper freeze-drying, their efficiency in protecting peptide agaist aggregation may be different (Corveleyn & Remon, 1996; Haque & Roos, 2006). Low-MW carbohydrates may be more efficient in immobilizing the peptides in their glassy structure. High-MW carbohydrate excipients, however, are prone to molecular steric hindrance (Kawai, Hagiwara, Takai, & Suzuki, 2004). As a result, maltodextrin may not be as efficient as lactose in stabilizing the antioxidant capacity of MPH during freeze-drying.

A biological assay for antioxidant capacity of reconstituted MPH prepared in lactose excipient was performed on HepG2 cells. Cell populations, grouped in different comet classes, are shown in **Fig. 15**. The majority (84.2%) of HepG2 cells before H_2O_2 exposure were originally ***Class 0*** (**Fig. 15a**). Exposure to H_2O_2 resulted in a reduction of ***Class 0*** cells and an increase in the populations of ***Class 3*** and ***Class 4*** to 51.9% and 14.7% of the population, respectively, suggesting dramatic DNA damage (**Fig. 15b**). The addition of MPH during cell exposure to H_2O_2 could not prevent DNA damage (**Fig. 15c**). Most of the cells (33.59% of the population) were ***Class 3***, followed by ***Class 1*** and ***Class 2***.

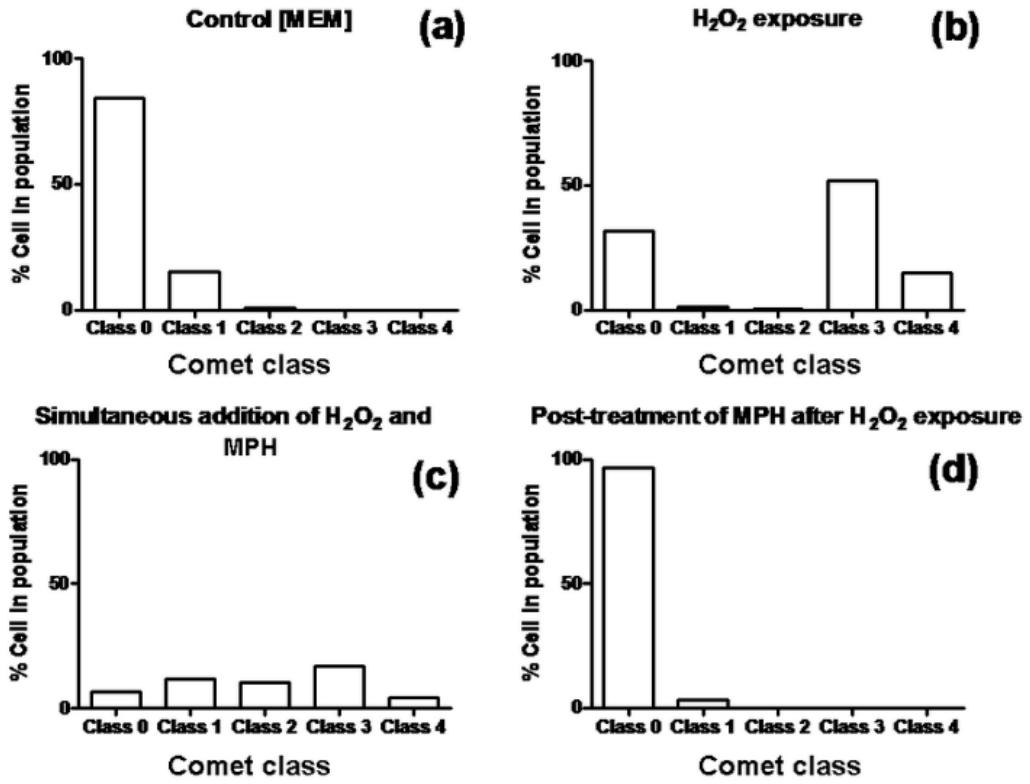

Fig. 15 Effect of mungbean protein hydrolysate (MPH) against oxidative stress of H_2O_2 on the % population of HepG2 cells. Genotoxicity was classified as comet classes: **Class 0** *(undamaged, no tail),* **Class 1** *(tail up to 1.5 times the diameter of comet nucleus),* **Class 2** *(tail 1.5–2.0 times the diameter of comet nucleus),* **Class 3** *(tail 2.0–2.5 times the diameter of comet nucleus) and* **Class 4** *(maximally damaged, tail >2.5 times the diameter of comet nucleus).*

Nevertheless, when MPH was taken up by HepG2 cells for 48 h after exposure to H_2O_2, the number of cells with DNA damage was reduced, and the vast majority of cells were ***Class 0*** (**Fig. 15d**), i.e. 96.7 % of the population. This was slightly higher than for cells grown in MEM media (**Fig. 15a**). Our results suggest the therapeutic activity of MPH on HepG2 against oxidative stress caused by H_2O_2.

The results of the biological assay suggested that MPH could enhance the efficacy of AA in HepG2. It is shown that in the presence of antioxidative MPH, the IC_{50} of AA was lowered from 58.5 to 38.5 μg/mL when MPH was present and co-dried with lactose excipient during freeze-drying (**Table 4**). Lactose-maltodextrin excipient, however, was not as effective as lactose excipient in lowering the IC_{50} of MPH-AA. Nonetheless, the level of AA uptaken, as well as its forms (i.e. molecular AA, AA micelle, or MPH-AA complex) would need systematic investigation to elucidate that the efficacy of AA was increased due to the influences of MPH in increasing the solubility of AA in aqueous phase; or the enhancement of MPH-AA penetration into HepG2 cells.

Table 4 Fifty percent inhibition concentration (IC_{50}) of asiatic acid (AA) on HepG2 using mungbean protein hydrolysate (MPH) as a carrier in different carbohydrate excipients.

Carbohydrate excipient co-dried with MPH and AA	**IC_{50} (μg/mL) of AA on HepG2**
AA acid alone (No excipient)	58.5[a]
Lactose	38.5[b]
Lactose:maltodextrin (1:1)	53.0[a]

Means followed by different superscripts are significantly different (P<0.05).

The different nature of MPH aggregation induced by different carbohydrate excipients; and the reduction in $ORAC_{FL}$ antioxidant capacity, caused by different carbohydrate excipients, may be responsible for the different efficacy of MPH-AA in HepG2 cell. In our study, there was no apparent change in the MWs of MPH after freeze-drying from MWs of more than 10.7 kDa, despite the addition of DTT. The aggregation of peptides and the reduction of $ORAC_{FL}$ in reconstituted MPH, induced by different carbohydrate excipients, may not involve the alterations of thiol groups or covalent bond formation in the large, soluble aggregates. Nevertheless, using carbohydrate excipients with different MW may induce depletion flocculation of the peptides in the presence of AA. The nature of surface-induced aggregation (Augustin & Hemar, 2009) of MPH by different weak forces is currently being investigated.

The ability of MPH-AA to lower the IC_{50} of AA on HepG2 may also open new pathways in exploring the potential use of bioactive food peptides and hydrolysates as cell-penetrating vehicles that can carry bioactive compounds, having a molecular structure close to pentacyclic triterpene, for specific purposes. Nevertheless, targeted-release delivery systems still require further in-depth investigation.

4. Conclusions

This study revealed for the first time that MPH prepared by tryptic hydrolysis of MPC had potential use as an antioxidative protein hydrolysate to increase the efficacy of pentacyclic triterpene anticancer AA. It was found that small carbohydrate molecules were more effective in maintaining $ORAC_{FL}$ antioxidant capacity after the freeze-drying process, and in lowering the IC_{50} of AA on HepG2. The interplay between bioactive compounds and nutrients suggested that food matrices may influence their bioavailability and bioactivity, both before and after dehydration. The effect of food processing and storage stability of bioactive compounds in dried powder form should thus be further investigated.

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3.2 Influence of carbohydrates on self-association of mungbean protein hydrolysate in the presence of amphiphilic asiatic acid

ABSTRACT

This study investigated the influence of surface-inactive carbohydrates on association characteristics of amphiphilic mungbean protein hydrolysate (MPH) and asiatic acid (AA) in aqueous suspension (11.72–11.94 % total solids). The carbohydrates investigated were trehalose, maltodextrin, mixed trehalose–maltodextrin, and mixed maltodextrin–starch. The presence of low-molecular-weight carbohydrates enhanced micellization of AA to form micrometre-sized particles due to depletion flocculation. Nonetheless, the presence of starch retained the sub-micrometre size of AA. In contrast, the presence of starch enhanced self-association of MPH *via* segregative phase separation. However, the mixed suspensions containing MPH, AA and carbohydrate in a ratio of 1:0–0.072:2.34, respectively, retained particle sizes of around 300 nm regardless of the carbohydrate used. It was found that the MPH–AA co-aggregates were stable against the osmotic effect of carbohydrates. The results suggest that carbohydrates regulated the aggregate size and surface hydrophobic region of MPH and MPH–AA by controlling surface-induced aggregation.

1. Introduction

Food proteins and their hydrolysates are not only used as energy-bearing macronutrients for humans, but also as biofunctional compounds, e.g. antioxidative, antihypertensive, and opioid compounds (Chen *et al.*, 2006; Augustin & Hemar, 2009). The studies on bioactive food peptides and protein hydrolysates from milk, meat, legumes, etc. have been investigated extensively on the influences of amino acid sequences on biofunctional properties through chemical assays (Samaranayaka & Li-Chan, 2011). Although enzymatic hydrolysis shortens the polypeptide chain and enhances the solubility of the hydrolysates, it could result in the self-association of small soluble peptides and soluble aggregates, thus altering the biofunctional properties of the hydrolysates (Kuipers *et al.*, 2006; Creusot & Gruppen, 2007). Factors affecting self-association of food proteins and peptides during hydrolysis and storage depend on conformation of proteins, amino acid composition, protein concentration, ionic strength, pH, hydrolysis process, bioactive molecules, and environmental alterations during storage of dried bioactive protein and peptide powders (Boulet *et al.*, 2000; van der Linden & Venema, 2007). Mungbean (*Vigna radiata* (L.) Wilczek) is an important source of protein for humans in Asia. It contains 19–28% protein (dry basis), and is rich in lysine but deficient in methionine and cysteine. The major proteins in mungbean seeds are vicilin (8S), followed by legumin (11S) and basic 7S globulin. Only the basic 7S and 11S have disulfide bonds (Tecson-Mendoza *et al.*, 2001). Mungbean protein isolate has many desirable functions in processed foods,

such as foaming, emulsifying, gelling, and water absorption (El-Adawy, 2000). Biofunctional properties of mungbean protein hydrolysate and peptides, such as antioxidant capacity and angiotensin-converting enzyme (ACE) inhibitory activity, have previously been reported (Shahidi & Zhong, 2008; Wongekalak *et al.*, 2011).

Our previous investigation indicated that different carbohydrate excipients used during drying of antioxidative mungbean protein hydrolysate (MPH)–asiatic acid (AA) resulted in different anticancer efficacy of AA in human hepatoblastoma HepG2 cells when MPH was used as a peptide carrier for AA. It was also found that the disaccharide lactose enhanced the cytotoxicity of MPH–AA co-aggregates compared with that of AA alone (Wongekalak *et al.*, 2011; Jirasripongpun *et al.*, 2012). The influence of different carbohydrates on the efficacy of MPH–AA for cellular uptake led to the suggestion that the molecular weight (MW) of the carbohydrate may influence the aggregation nature of AA and MPH–AA. This, in turn, could result in different particle sizes of AA and MPH–AA readily for cellular uptake.

Carbohydrates such as lactose, sucrose, trehalose, maltodextrin, starch, and hydrocolloids have been used as excipients to enhance the stability of bioactive compounds during drying and storage (Gibbs *et al.*, 1999; Andya *et al.*, 2003; Wang, 2005; Zhang *et al.*, 2007). These carbohydrates have been used as stabilisers for bioactives during mixing, drying, and storing due to their high glass transition temperature (T_g), which helps lower the molecular mobility of bioactives during storage (Augustin & Hemar, 2009). However, carbohydrates can induce depletion flocculation of protein molecules by increasing the osmotic pressure of water (McClements, 2000, 2006). They can thus alter the association characteristics of colloidal peptides (Singh & Lillard, 2009). Nevertheless, investigation of the interplay among surface-active molecules and surface-inactive molecules such as carbohydrates on the aggregation characteristics of colloidal particles has been limited.

AA is a pentacyclic triterpene having amphiphilic characteristics, and is prone to micellization in the aqueous phase due to its low hydrophile–lipophile balance (HLB) (Rafat *et al.*, 2008). It was hypothesized that the surface behaviour of AA and MPH could be altered in the presence of carbohydrates having different MWs and viscosities due to the changes in osmotic pressure and the nature of surface-induced associations of both surface-active molecules.
The objectives of the present study were to investigate the association characteristics of MPH and AA in the absence or presence of surface-inactive carbohydrates. The insights may help better understand the influence of carbohydrates on surface-induced aggregation of surface-active compounds like food peptides and amphiphilic pentacyclic triterpene AA, which have a molecular structure similar to that of bile acid.

2. Materials and Methods

Materials

De-hulled mungbeans (Raithip, Bangkok, Thailand) were purchased from a local supermarket. Trypsin (EC 3.4.21.4; 10,000 BAEE U mg^{-1}), asiatic acid (AA), and fluorescein (Na salt) were purchased from Sigma-Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid), potassium persulfate, 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH), and 1-anilino-8-naphthalenesulfonate (ANS) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Fluka (Buchs, Switzerland). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate dihydrate, and sodium chloride were purchased from Ajax Finechem (Seven Hills, NSW, Australia). Cassava maltodextrin with dextrose equivalent (DE) of 18 (Neo-Maldex®) was purchased from Neotech Food Co. (Ratchaburi, Thailand). Trehalose (Treha™; Hayashibara, Okayama, Japan) and pre-gelatinized cassava starch were provided by EAC Thailand and Siam Modified Starch (Bangkok, Thailand), respectively.

Effect of tryptic hydrolysis process on physicochemical properties of mungbean protein concentrate (MPC) and mungbean protein hydrolysate (MPH)

De-hulled mungbeans, which were kept at room temperature of 27 °C in sealed plastic bagfor less than one month after manufacturing, were milled in a Mikro-Pulverizer® (Hosokawa Micron Group, Osaka, Japan) to obtain mungbean flour. The flour suspension (6.67 % w/v) was prepared in distilled water and adjusted to pH 8.0 using 2 M NaOH to extract proteins. The suspension was continuously stirred for 1 h at 25 °C and centrifuged at 14000 *g* (Sorvall RC-5C Plus; DuPont, Wilmington, DE, USA) at 15 °C for 30 min. Spent flour was discarded. The supernatant was heated at 80 °C for 30 min to inactivate trypsin inhibitor and then cooled to room temperature. Five mM $NaHSO_3$ and 5 mM $CaCl_2$ were added and the pH was adjusted to 4.5 using 2 M HCl. The suspension was kept at 4 °C for 2 h and centrifuged at 9000 *g* at 4 °C for 15 min. The supernatant was discarded and mungbean protein concentrate (MPC) was dried in a tray dryer (KAN 1960; Kan Seng Lee Machinery, Bangkok, Thailand) at 60 °C for 6 h and kept at −20 °C before use. The protein content was determined by Kjeldahl method (AOAC, 1996).

Mungbean protein hydrolysate (MPH) was prepared by re-suspending the MPC in distilled water to obtain 4% protein (w/v), and adjusting the pH to 8.0 using 2 M NaOH. The proteins were hydrolysed by trypsin at 37 °C for 0, 30, 60, and 90 min using a mungbean protein-to-enzyme ratio of 31:1. The

enzymatic reaction was inactivated by diluting the suspensions with appropriate diluents for further analyses, as described below.

MWs of mungbean proteins and peptides during tryptic hydrolysis

The MWs of MPC and fresh hydrolysates were characterized by tricine SDS-PAGE separating gel (Schägger & Jagow, 1987). The continuous buffer contained 3 mM Tris-HCl (pH 8.45) and 0.3% sodium dodecyl sulfate (SDS) in separating gel and stacking gel. The anode running buffer (outside) consisted of 0.02 M Tris-HCl, pH 8.9; while the cathode running buffer (inside) contained 0.1 M Tris, 0.1 M tricine, and 0.1% SDS. The samples were added to dissociating buffer (0.5 M Tris-HCl [pH 6.8], 5.5% SDS, 7% glycerol, and 0.01% Coomassie brilliant blue R-250 with or without 2% dithiothreitol [DTT]). Each solution was heated at 100 °C for 4 min, cooled, and centrifuged at 2125 *g* (Spectrafuge™ 16M; Labnet International, Edison, NJ, USA) for 5 min to remove insoluble material. An aliquot of the sample solution containing 0.02 mg protein or 5 µL low-range MW standards was loaded into each well. Electrophoresis was run at a constant current of 35 mA for 4 h. Gel slabs were fixed and stained simultaneously using Bio-Rad Coomassie blue R-250 stain solution (10% acetic acid, 0.025% Coomassie blue R-250) for 2 h, and then de-stained by 10% acetic acid.

Size distribution and ζ- potential

Size distribution and ζ- potential of MPC and MPH for hydrolysis times of 0, 30, 60, and 90 min were measured by diluting the mixture solution using filtered (0.22 µm; Millipore, Billerica, MA, USA) deionized water to obtain a final protein concentration of 0.14% w/v. They were analyzed by a Zetasizer (model ZEN3600; Malvern Instruments, Malvern, Worcestershire, UK) connected to an autotitrator (model MPT-2; Malvern Instruments) under flow condition. The solutions were mixed continuously by magnetic stirring.

Reversibility of MPH as a function of pH

Size distribution and ζ- potential of MPH after hydrolysis for 30 min were measured by diluting the mixture solution using filtered deionized water, as described above, using a Zetasizer connected to an autotitrator. Titration was performed by acidification to pH 2.0 using 1 M HCl and alkalization from pH 2.0 to pH 8.0 using 1 M NaOH.

Surface hydrophobicity (So)

The samples were analysed for surface hydrophobicity (*So*) using 1-anilino-8-naphthalenesulfonate (ANS magnesium salt), as described by Hayakawa & Nakai (1985), to determine hydrophobic region mainly composed of aromatic ring. Samples were diluted to 0.008–0.030% protein (w/v) with 0.01 M phosphate buffer, pH 7.0. Two mL of each sample was mixed with 10 µL of 8.0 mM ANS in 0.1 M phosphate buffer, pH 7.0, and allowed to stand for 20 min. Two hundred µL of sample, with or without ANS, was injected into a microplate reader (Infinite® M200 Pro; Tecan Group, Männedorf, Switzerland). Fluorescence intensity was measured under the following conditions: λ_{ex} = 390 nm (excitation bandwidth = 9 nm), λ_{em} = 470 nm (emission bandwidth = 20 nm), gain 103. Each slope represented surface hydrophobicity value, and was calculated by least squares analysis.

Trolox equivalent antioxidant capacity (TEAC) assay

Freshly prepared MPC and MPH suspensions were assayed for Trolox equivalent antioxidant capacity (TEAC) (Re *et al.*, 1999; Sun *et al.*, 2007). The TEAC assay determined electron transfer reaction (Huang, Ou, & Prior, 2005). Briefly, $ABTS^{\bullet+}$ radical solution was prepared by mixing 20 mM ABTS and 70 mM $K_2S_2O_8$ in the dark for 16 h at room temperature. The solution was diluted before use with PBS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, and 153.84 mM NaCl) to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Thirty µL of MPC or MPH (1.27% protein) was mixed with 3 mL of $ABTS^{\bullet+}$ radical solution and incubated at 30 °C for 4 min. The absorbance at 734 nm was recorded using a spectrophotometer (Spectronic 20+; Thermo Fisher Scientific, Waltham, MA, USA). The calibration curve was prepared using Trolox concentrations ranging from 0.1 to 2 mM. TEAC values were calculated by plotting the absorbance of decolourized $ABTS^{\bullet+}$ radical solution *vs.* concentration of Trolox. The antioxidant capacity of samples was expressed as µmol Trolox equivalent per 1 mg of protein in MPC or MPH (µmol TE mg^{-1} protein).

Oxygen radical absorbance capacity–fluorescein ($ORAC_{FL}$) assay

The $ORAC_{FL}$ assay measured hydrogen atom transfer reaction (Huang *et al.*, 2005). $ORAC_{FL}$ assays (Prior *et al.*, 2003) were carried out on a Tecan microplate reader (Infinite M200 Pro). The reaction was performed at 37 °C in 75 mM phosphate buffer (pH 7.4); the final reaction mixture was 200 µL. Antioxidant (20 µL; Trolox 2–9 µM, or sample 1–3 mg mL^{-1}) and fluorescein (120 µL; 70 nM final concentration) were loaded into a black 96-well microplate. Fluorescence intensity was measured under the following conditions: the temperature of the incubator was set to 37 °C; a fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was

programmed to record the fluorescence of fluorescein once per cycle. The number of cycles was 70 cycles, each cycle was run for 2 min and 36 s with the time lapse between cycles of 0 μs. During cycle four, 60 μL of AAPH was manually injected into the respective wells to give a final AAPH concentration of 12 mM. The plate contents were mixed by shaking for 1 s before reading. The final $ORAC_{FL}$ values were calculated by using a linear equation between the Trolox standards or sample concentrations and net areas under the fluorescein decay curves. The area under the curve (AUC) was calculated by:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 +, ..., + f_i/f_4) \times CT$$

where f_4 is the initial fluorescence reading at cycle 4, f_i is the fluorescence reading at cycle i, and CT is the cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of a sample. The $ORAC_{FL}$ values of samples were then reported as μmol Trolox equivalent per mg of protein (μmol TE mg^{-1} protein).

Effect of AA concentration on the degree of MPH–AA aggregation

One mL of MPH (3.6% w/v protein) after 30 min tryptic hydrolysis was mixed with 25 μL of AA (0–20.4% w/v in 99% ethanol) to obtain different concentrations of 0–0.5% w/v AA. The suspensions were further diluted with distilled water to obtain 0.14% w/v protein and 0–0.03% w/v AA, and were measured for absorbance at 400 nm (Abs_{400}) using a Genesys 10S UV-vis spectrophotometer (Thermo Fisher Scientific). The degree of aggregation was reported as turbidity (Pearce & Kinsella, 1978), calculated as:

$$Turbidity = \frac{Abs_{400}}{Pathlength\ (cm)} * 2.303$$

Mixed suspensions of MPH–AA were characterized for size distribution, ζ-potential, surface hydrophobicity, and antioxidant capacity using the methods described above.

Effect of carbohydrate type on the aggregation of MPH–AA

The MPH suspension obtained after 30 min tryptic hydrolysis was mixed with AA (10.2% w/v in 99% EtOH), the core material, in the presence of carbohydrate excipients – namely, trehalose (T); mixed trehalose and maltodextrin at a ratio of 1:1 (TM); maltodextrin (M); and mixed maltodextrin and starch at a ratio of 9:1 (MS) – using a protein: AA: carbohydrate ratio of 1: 0–0.072: 2.34. Final suspensions (total solids of 11.72–11.94% w/v) contained 3.47% protein (w/v), 0–0.22% AA (w/v), and

8.14% carbohydrate (w/v). They were evaluated for size distribution, surface hydrophobicity, and $ORAC_{FL}$ using the methods described above.

Statistical Analysis

The mixtures were prepared in two separate trials. The data were analysed by analysis of variance (ANOVA) with significance at $P < 0.05$. Significant differences among mean values were determined by Duncan's multiple range test. All statistical analyses were performed using SPSS software version 12.

Results and Discussion

Effect of tryptic hydrolysis on physicochemical properties of MPC and MPH

Mungbean proteins had MWs above 16.9 kDa prior to hydrolysis (**Fig. 16**). However, the MWs of proteins in the hydrolysates decreased during hydrolysis. Most mungbean peptides had MWs below 16.9 kDa after being hydrolyzed for 30 min. Under reducing condition by DTT, there was no observable change in the MWs of the peptides in MPH.

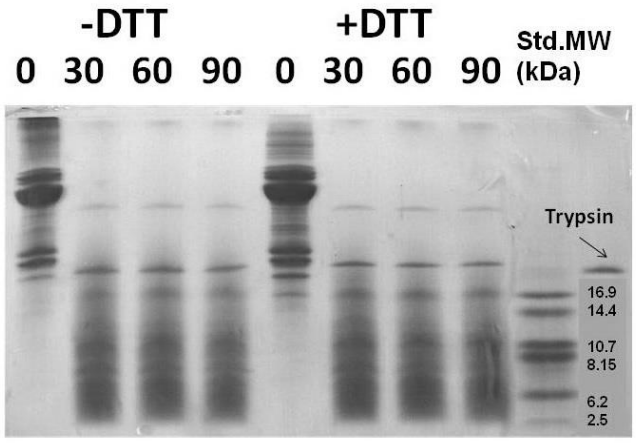

Fig. 16 Effect of hydrolysis time in minutes on MWs of mungbean proteins and peptides.

After tryptic hydrolysis for 30 to 90 min, the length-scale size of mungbean peptides was reduced compared with unhydrolyzed MPC (**Table 5**). Prior to hydrolysis, the MPC suspensions had a wide range of particle size. The ζ-potential of the suspensions significantly increased during hydrolysis due to the liberation of small peptides ($P < 0.05$) (**Table 5**), suggesting more exposed charged groups of the peptides during hydrolysis. The surface charge of the peptides was higher than (-)30 mV, resulting in the ability of mungbean peptides to repel each other and inhibit their self-association in MPH. The ANS surface hydrophobicity of MPH was significantly lower than that of MPC ($P < 0.05$). After tryptic hydrolysis, the $ORAC_{FL}$ and TEAC values of MPH were significantly increased ($P < 0.05$).

Table 5 Physicochemical characteristics of mungbean proteins during tryptic hydrolysis

Time (min)	ζ- potential (mV)	Surface hydrophobicity ($So \times 10^5$)	Antioxidant capacity (μmol TE mg^{-1} protein)	
			$ORAC_{FL}$ assay	TEAC assay
0	(-)16.98^{b} ± 3.55	12.50^{a} ± 5.00	0.210^{b} ± 0.013	0.453^{c} ± 0.000
30	(-)33.20^{a} ± 0.96	6.22^{b} ± 1.14	0.706^{a} ± 0.102	1.404^{a} ± 0.459
60	(-)32.65^{a} ± 1.53	4.90^{b} ± 0.37	0.784^{a} ± 0.175	0.878^{b} ± 0.305
90	(-)32.85^{a} ± 1.53	4.97^{b} ± 0.48	0.662^{a} ± 0.072	0.759^{bc} ± 0.281

Means ± SD in the same column followed by different superscripts are significantly different ($P < 0.05$)

Acidification of MPH from the actual pH after hydrolysis to pH 2.0 (to inactivate trypsin) and alkalization from pH 2 to pH 8 affected the Z-average size of the peptides (**Fig. 17a**). Mungbean peptides reversibly associated to large-sized aggregates in the isoelectric pH range (pI) of 4.2 (**Fig. 17b**). Although the MPH aggregates had different particle sizes, the surface charges of the peptides remained the same when the pH was shifted during acidification and alkalization. It should be noted that the negative surface charge of MPH at neutral pH was high enough to prevent aggregation of peptides.

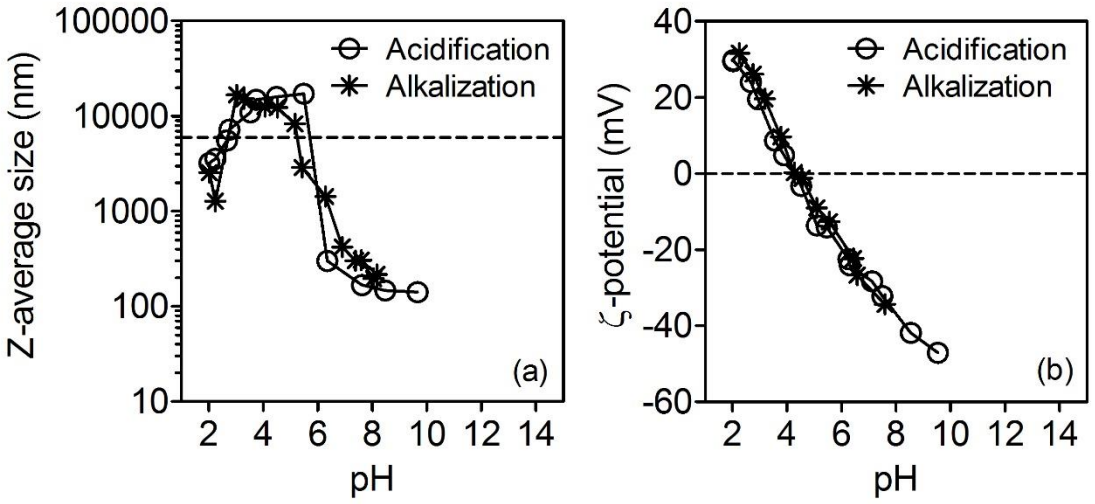

Fig. 17 Effect of acidification and alkalization on (a) Z-average size and (b) ζ-potential of mungbean peptide aggregates.

Effect of AA concentration on the aggregation of MPH–AA

The concentration of AA influenced the aggregation of colloidal particles in MPH–AA mixed suspensions (**Fig. 18**). At a low concentration of AA, raising the AA content increased the turbidity of MPH–AA mixed suspensions (**Fig. 18a**). At a high concentration of AA, i.e. above 0.1 g AA in 1.0 g protein, the turbidity of mixed suspensions remained constant. Most of the aggregates in mixed suspensions were 200 nm in size (**Fig. 18b**) when the ratios of AA to protein in MPH were 0:1 and 0.003:1 (w/w) (**Fig. 18b**).

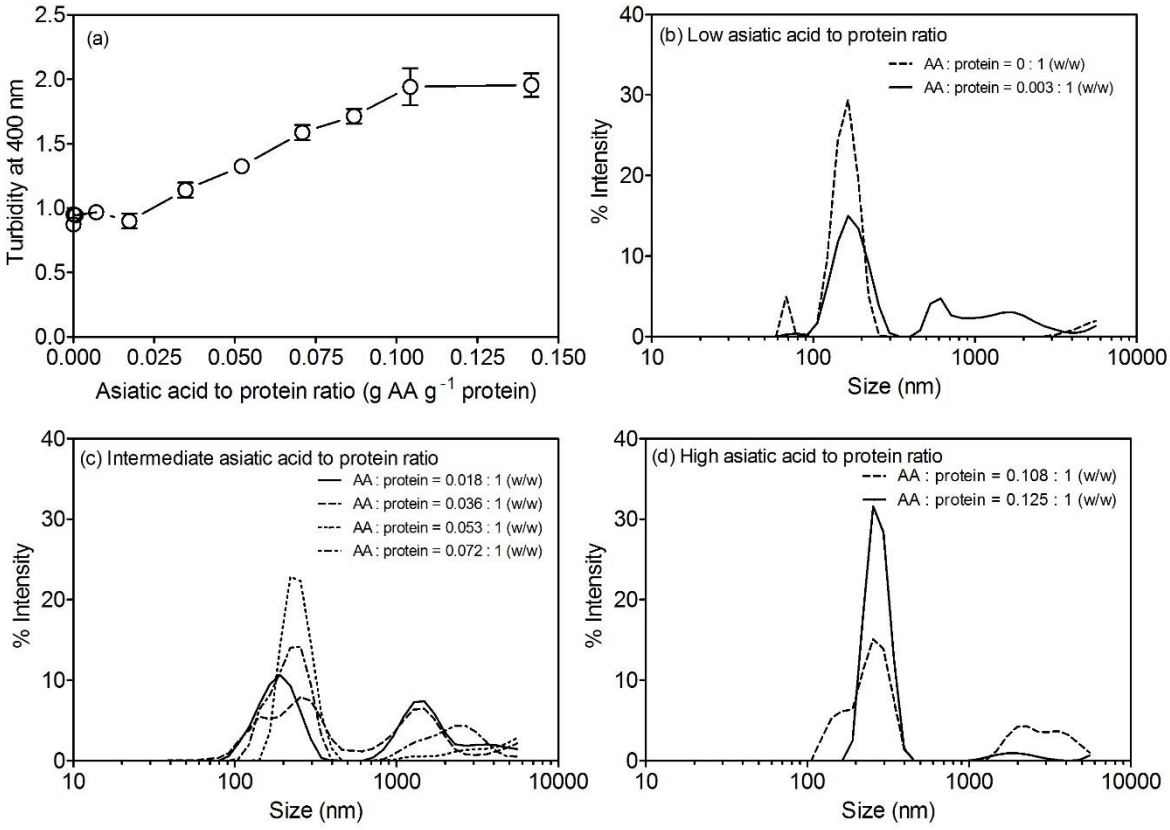

Fig. 18 Effect of AA to protein ratios on the size of particles in MPH–AA mixed suspensions, measured as (a) turbidity at 400 nm, and (b–d) size distribution. The mixed suspensions were prepared in distilled water containing total solid concentration of 0.14% protein and 0–0.03% AA (w/v) for turbidity measurement. For size distribution, the mixed suspensions were prepared in double-deionized water and contained 0.12% protein and 0–0.014% AA (w/v).

When the concentration of AA was increased from a ratio of AA to peptide in MPH of 0.018: 1 to 0.072: 1 (w/w), the turbidity increased linearly and the majority of particles in the mixed suspension had particle sizes of around 300 nm (**Fig. 18c**). Nonetheless, it appeared that the mixed suspensions possessed colloidal particles having bimodal distribution (300 nm *vs.* 2,000 nm at the same % intensity) when the ratios of AA to peptide were 0.018: 1 and 0.036: 1. Above the ratios of 0.53: 1 and 0.072: 1, the majority of colloidal particles remained at a small size of around 300 nm, although the micrometre size existed at less % intensity. At ratios of AA to peptide of 0.108: 1 to 0.140: 1 (w/w), the turbidity remained constant. However, an AA-to-peptide ratio up to 0.125: 1 would be required to obtain a monomodal distribution of particles with particle sizes of less than 400 nm (**Fig. 18d**). These results suggested that the association nature of MPH and AA was dependent on the mass ratio of AA to peptide in MPH.

AA in the aqueous phase had a Z-average size around 609–769 nm at a concentration between 0.004 to 0.014% (w/v) due to micellization or self-association of the amphiphilic structure. Increasing the concentration of AA in water induced micellization *via* hydrophobic interactions, observed as an increase in surface hydrophobicity (**Table 6**). AA is an anionic amphiphilic molecule, which has a low HLB of 5.5 and a critical micelle concentration (CMC) around 16 μM, or 0.00008% (w/v), when dispersed in water (Rafat *et al.*, 2008). Increasing the AA concentration to the range investigated in the present study enhanced self-association mainly *via* hydrophobic contact (Rafat *et al.*, 2008; Stephenson *et al.*, 2008).

Table 6 Effect of AA concentration on physicochemical characteristics of mixed MPH-AA suspensions in aqueous phase

AA suspension			MPH-AA mixed suspension		
Concn. of AA (% w/v)	Z-average size (nm)	*So* (x 10^5)	AA: MPH ratio at corresponding AA concn. (% w/v)	Z-average size (nm)	*So* (x 10^5)
0.000	ND	ND	0.000: 1	180^{b} ± 30	6.72^{a} ± 0.07
0.0003	ND	0.012^{d} ± 0.000	0.003: 1	243^{a} ± 43	6.76^{a} ± 0.02
0.002	ND	0.009^{d} ± 0.001	0.018: 1	233^{ab}± 42	6.63^{a} ± 0.04
0.004	609^{a} ± 81	0.048^{d} ± 0.002	0.036: 1	233^{ab}± 22	6.65^{a} ± 0.03
0.006	471^{a} ± 33	0.175^{cd}± 0.013	0.053: 1	264^{a} ± 43	5.43^{a} ± 0.07
0.008	847^{a} ± 95	0.524^{c} ± 0.019	0.072: 1	278^{a} ± 69	6.36^{a} ± 0.01
0.012	733^{a} ± 30	1.335^{b} ± 0.003	0.108: 1	278^{a} ± 6	8.26^{a} ± 0.13
0.014	769^{a} ± 143	2.280^{a} ± 0.063	0.125: 1	242^{a} ± 21	7.48^{a} ± 0.16

Means ± SD in the same column followed by different superscripts are significantly different ($P < 0.05$). ND = Not detected.

The Z-average size of MPH was 180 nm, which was slightly increased to around 240 nm in the presence of AA (**Table 6**). These results indicated that MPH–AA co-aggregates could be formed, resulting in the disappearance of micellar AA. Both monomodal and bimodal distributions of colloidal particles in mixed suspensions showed concentration-dependent association. This suggested both MPH–AA and MPH associations. The presence of MPH prevented self-association of AA and improved dispersibility of AA in water at corresponding AA concentration. However, there was no observable change in surface hydrophobicity of MPH–AA compared to that of MPH. This is probably because the magnitudes of *So* of MPH and MPH–AA aggregates were much higher than that of micellar AA (**Table 6**).

AA has three hydroxyl groups and one carboxylic group in the hydrophilic region, and pentacyclic triterpene with methyl groups in the hydrophobic part (Rafat *et al.*, 2008). The structure of AA is thus close to that of bile acid. This, in turn, could help induce MPH–AA association *via* electrostatic interaction between carboxylic groups of AA and NH_2 groups of lysine and arginine generated after

tryptic hydrolysis, as well as *via* H-bonding (Semenova, 2007), hydrophobic interactions, and van der Waals forces (Roda *et al.*, 1982).

Effect of carbohydrate excipients on aggregation of MPH-AA

Table 7 shows the effect of carbohydrate excipients on Z-average size of AA, MPH and MPH–AA co-aggregates prepared at a protein: AA: carbohydrate ratio of 1: 0.072: 2.34. The presence of carbohydrate in the aqueous phase increased the Z-average size of AA. Trehalose (T), maltodextrin (M), and their mixture enhanced micellization of AA, resulting in Z-average sizes of 1,057, 922 and 947 nm ($P \geq 0.05$), respectively. However, such surface-induced association of AA by carbohydrates did not involve hydrophobic interactions, measured by ANS hydrophobicity, among AA molecules.

Table 7 Effect of carbohydrate types on Z-average size of colloidal particles in mixed MPH-AA-carbohydrate. The ratio between MPH:AA:carbohydrate was 1:0.072:2.34 and total solid concentration in double deionized water was 0.40 % w/v.

Carbohydrate excipient	Z-average size (nm)			Surface hydrophobicity (S_o x 10^5)		
	AA alone	MPH	MPH-AA	AA alone	MPH	MPH-AA
No excipient	$586^{b} \pm 25$	$180^{b} \pm 30$	$278^{a} \pm 69$	$0.52^{a} \pm 0.02$	$6.22^{a} \pm 0.61$	$6.50^{a} \pm 0.02$
Trehalose (T)	$1057^{a} \pm 352$	$223^{b} \pm 40$	$240^{a} \pm 18$	$0.33^{a} \pm 0.00$	$5.75^{ab} \pm 0.76$	$6.42^{a} \pm 0.34$
Trehalose-maltodextrin (TM)	$947^{a} \pm 192$	$241^{b} \pm 53$	$326^{a} \pm 62$	$0.34^{a} \pm 0.06$	$4.97^{ab} \pm 0.29$	$5.57^{ab} \pm 1.90$
Maltodextrin (M)	$922^{a} \pm 26$	$263^{ab} \pm 40$	$298^{a} \pm 73$	$0.42^{a} \pm 0.07$	$5.56^{ab} \pm 0.55$	$6.34^{a} \pm 1.20$
Maltodextrin-starch (MS)	$418^{b} \pm 76$	$338^{a} \pm 81$	$287^{a} \pm 4$	$0.42^{a} \pm 0.12$	$4.37^{b} \pm 0.24$	$4.98^{ab} \pm 0.32$

Means ± SD in the same column followed by different superscripts are significantly different ($P < 0.05$).

Maltodextrin–starch mixture (MS), however, helped restrict the Z-average size of AA to 418 nm, similar to that in the absence of carbohydrate. The higher viscosity of starch may help in maintaining the degree of AA micellization by retarding the Brownian motion of AA in the aqueous phase.

Carbohydrates also enhanced the association of peptides in MPH. A low-MW carbohydrate such as trehalose was less effective than maltodextrin or a maltodextrin–starch mixture in inducing MPH aggregation. It is possible that the negative charge of MPH was high enough to reduce the osmotic effect of trehalose and a mixture of trehalose–maltodextrin. Self-association of MPH was promoted only in the presence of high-MW carbohydrates. The presence of starch enhanced the self-association of MPH nanoclusters into large-size aggregates. This was probably due to the high volume ratio of starch, which induced mutual exclusion of peptides and carbohydrates through thermodynamic incompatibility, or segregative phase separation, of peptide phase and carbohydrate phase (McClements, 2000; Mahler *et al.*, 2009; Philo & Arakawa, 2009).

The influence of carbohydrates on surface association of AA or MPH was, however, insignificant in MPH–AA mixtures ($P \geq 0.05$). It should be noted that the Z-average size of MPH–AA in different carbohydrate suspensions remained within 240–326 nm, regardless of carbohydrate type (Table 3). The surface nature of MPH–AA co-aggregates could differ from that of micellar AA and MPH aggregates, making them less prone to further association induced by carbohydrates. Thus, the size of MPH–AA particles remained small, within sub-micrometre size.

The surface hydrophobicities of MPH and MPH–AA were much higher than that of AA alone, regardless of carbohydrate type (**Table 7**). The MPH–AA co-aggregates were quite stable against depletion flocculation and segregative phase separation in terms of Z-average size and hydrophobic interactions. In addition, carbohydrates did not significantly affect the antioxidant capacity of MPH and MPH–AA co-aggregates, as measured by $ORAC_{FL}$ assay ($P \geq 0.05$) (**Table 8**). These results suggested that the presence of AA and all carbohydrates did not affect the reactive groups responsible for the antioxidant capacity of peptides in MPH.

Table 8 Effect of carbohydrate excipients on $ORAC_{FL}$ antioxidant capacity of MPH and MPH-AA mixture

MPH-AA mixture in different excipient	$ORAC_{FL}$ (μmol TE mg^{-1} protein) [ns]	
	MPH	MPH-AA
No excipient	0.583 ± 0.106	0.495 ± 0.122
Trehalose (T)	0.587 ± 0.104	0.515 ± 0.054
Trehalose-maltodextrin (TM)	0.593 ± 0.020	0.471 ± 0.063
Maltodextrin (M)	0.559 ± 0.049	0.561 ± 0.012
Maltodextrin-starch (MS)	0.564 ± 0.095	0.621 ± 0.107

[ns] Indicates no significant difference ($P \geq 0.05$).

This study showed that antioxidative MPH from tryptic hydrolysis, which had a fairly high surface charge of (-)30 mV, could reduce micellization of AA in the aqueous phase. The MPH–AA co-aggregates may help to increase cellular uptake *via* passive diffusion by controlling the particle size to around 300 nm (Wongekalak *et al.*, 2011; Jirasripongpun *et al.*, 2012). The degree of co-aggregation between MPH and AA was found to be dependent on their ratio and the type of carbohydrate in the bulk aqueous phase. The presence of a carbohydrate could regulate the aggregate size and surface hydrophobic regions of MPH and MPH–AA by influencing surface-induced aggregation *via* depletion flocculation of the colloidal particles. Nevertheless, the presence of starch could control the MPH aggregate size by retarding Brownian motion due to the high viscosity. However, the surface behaviours of AA and MPH during oral ingestion, gastric digestion, and intestinal digestion under the physiological conditions of the gastrointestinal tract require further investigation.

Conclusions

This study showed the association characteristics of MPH and AA in the absence or presence of surface-inactive carbohydrates. The sizes of the aggregates, as well as the surface hydrophobic region of MPH, were controlled by depletion flocculation due to the presence of carbohydrates. The MPH–AA co-aggregates could endure the osmotic gradient effect of carbohydrates. A more thorough understanding of the interplay among amphiphilic molecules and surface-inactive carbohydrates could help better control the biofunctionalities of peptide-based encapsulation in amorphous carbohydrate excipient for dry products.

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บทที่ 4 การเปลี่ยนแปลงของโปรตีนไฮโดรไลเสทระหว่างการเก็บรักษาและสภาวะการย่อย

4.1 Excipient influences on moisture-induced aggregation of spray-dried whey protein and tryptically hydrolyzed whey protein powder

Abstract

Excipients trehalose (TH) and maltodextrin (MD) were mixed with whey protein concentrate (WPC) and whey protein hydrolysate (WPH) using the protein to excipient ratio of 0.3: 0.7 and spray-dried. The glass transition temperature (Tg) of WPC-TH, WPC-MD, WPH-TH and WPH-MD powder before storage was in the range of 60 – 90 ^{o}C depending on the sources of protein and the types of excipient. Fluorescence quenching showed that fluorophore – site accessibility of whey proteins embedded in MD was higher than those in TH excipient. After storage at 24% RH at 35 ^{o}C, the Tg of WPC-TH was reduced to 28 ^{o}C and moisture-induced aggregation of whey proteins occurred as revealed by 2-dimensional gel electrophoresis. MD excipient, however, prevented moisture-induced aggregation of whey proteins during storage and retained thiol group content of WPC-MD and WPH-MD compared to those before storage. Structural transition of whey proteins in the powder form was influenced by excipient.

1. Introduction

The solid form of a controlled delivery for therapeutic or bioactive proteins such as human serum albumin, hormones, vaccine and enzymes yields many advantage over the liquid form, particularly in practical handling. However, the increase in the protein concentration during dehydration process, which brings the proteins to the close proximity, may limit the biological activities of the bioactive proteins due to their unfolding and aggregation during dehydration and storage.

Dried therapeutic or bioactive proteins in pharmaceutical industry often contain carbohydrate excipients to protect proteins in the solid state during drying and storage (Mensink, Frijlink, Maarschalk, & Hinrichs, 2017). When a protein suspension is dried in the presence of sugar or carbohydrates, which can range from 5 to 50 % when starch or glucose syrup is used as pharmaceutical excipients, the amorphous matrices of carbohydrate are formed and embed protein molecules within the matrices. Subsequently, the embedded protein molecules in the amorphous matrices are stable against conformational changes during dehydration and storage since the

interactions between protein-protein are limited (Imamura, Ogawa, Takaharu, Sakiyama, & Nakanishi, 2003).

Currently whey protein hydrolysate is considered as important biofunctional ingredient that provide antioxidative, antihypertensive, antimicrobial, antiproliferative and immunomodulatory activities (FitzGerald, Murray, & Walsh, 2004; Shahidi & Zhong, 2008). In addition, we have recently reported the use of reconstituted antioxidative whey protein hydrolysate powder obtained from hydrolysis of commercial whey protein concentrate (WPC) by trypsin for 60 min (Hongsprabhas, Kerdchouay, & Sakulsom, 2011) or pepsin for 60 min (Rongsirikul & Hongsprabhas, 2016), as an antibrowning agent in thermally processed foods. The mechanisms of antibrowning of hydrolysed WPC involved the delay of fluorescence advanced glycation end product (MRP) formation in sterilized solutions containing reducing sugar-glycine or reducing sugar-WPC. The tryptically hydrolysed WPC also showed efficacy in the prevention of MRP cytotoxicity in normal human intestinal FHs 74 Int cells and human epithelial colorectal carcinoma Caco-2 cells after reconstitution.

Zhou, Liu, & Labuza (2008a, 2008b) demonstrated that short chain bioactive peptides in the powder in the absence of excipient are likely prone to aggregation during storage due to their small MWs, particularly when the powder has low glass transition temperature (*Tg*). This could make bioactive whey protein hydrolysates and peptides unstable to solid deterioration, leading to the reduction in bioactivities of protein hydrolysates and peptides during storage. Zhou, Liu, Chen, & Labuza (2014) also demonstrated that whey protein hydrolysates having high degree of hydrolysis (DH) were sensitive to moisture-induced aggregation more than those with low DH due to the presence of small MW peptides and amino acids. The sorbed water during storage can also worsen the aggregation, leading to the loss in solubility of bioactive proteins and peptides. The mechanisms responsible for irreversible protein aggregation in solid deterioration of therapeutic proteins include sulfhydryl-disulfide interchange (SH-SS interchange), β-elimination followed by thiol-catalysed disulfide bond formation, formaldehyde-mediated pathway, etc. (Costantino, Langer, & Klibanov, 1994; 1995; Liu, Langer, & Klibanov, 1991; Schwendeman, Costantino, Gupta, Siber, Klibanov, & Langer, 1995).

Although carbohydrates have been used as glassy matrices embedding the bioactive and therapeutic proteins, the presence of small MW peptides and amino acids in formulations could have detrimental effects on the storage stability of food protein hydrolysate powders having high degree hydrolysis (Zhou et al., 2014). The high content of sugar, for example lactose, can initiate lactose-induced stickiness in dairy powder (Hogan & O'Callaghan, 2010). Apparently the stabilizing mechanism of carbohydrate excipient to delay deterioration of bioactive or therapeutic proteins in solid state varies and needs further investigation since it is dependent on the types of protein, the

types of excipient, the formulation, as well as drying and storage conditions that determine protein degradation mechanisms in the solid state.

This study explored the storage stability of tryptically hydrolysed WPC co-dried with carbohydrate excipients. It was hypothesized that using different types of carbohydrate, namely trehalose (TH) and cassava maltodextrin (MD) could help stabilizing the powder containing whey proteins and whey protein hydrolysate. The glass transition temperature range of TH was 107 – 119 °C (Simperler et al. 2006) and that of maltodextrin was 112 – 142 °C (Avaltroni, Bouquerand, & Normand, 2004). However, both carbohydrate excipients had different molecular weights (MWs) and reducing properties. This could lead to different mechanisms in powder stabilization. The high MW carbohydrate excipient can reduce mobility of specific groups of the protein more effectively than the small MW sugar. The insights from this investigation could help understanding the interplay between whey protein, short chain peptide and carbohydrate excipient in storage stabilization of the powder.

2. Materials and methods

2.1 Materials

Commercial WPC was imported and repacked by a local distributor in Thailand. It contained 75.83 % protein (w.b.), 6.5 % moisture content (w.b.), 2.71 % ash (w.b.), 1.05% fat (w.b.) and 13.91% carbohydrate (w.b.) (AOAC, 2000). Trypsin (EC 3.4.21.4; 10,000 BAEE Umg^{-1}) and fluorescein (Na salt) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Trolox (6–hydroxy-2,5,7,8-tetramethylchroman–2–carboxylic acid), potassium persulfate and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Cheme (Steinheim, Germany). Trehalose (TH) was kindly supported by the East Asiatic Company Ltd., Bangkok, Thailand and cassava maltodextrin DE 18 (MD) was purchased from Neotech Food Co., Ltd., Rajburi, Thailand). Cyanidin-3-O-glucoside (C3G) was purchased from Polyphenol Laboratories AS (Sandnes, Norway).

2.2 Preparation of spray-dried whey protein and hydrolysate powder

Whey protein hydrolysate was prepared using protein to trypsin ratio of 100:1 as described previously (Hongsprabhas et al., 2011). The tryptic hydrolysis was allowed to proceed for 1 h at 37°C and trypsin was inactivated by adjusting the pH to 2.0 using 5 N HCl. Spray-dried powder of WPC and WPH in different excipients was prepared by mixing protein suspensions (5.69 % protein w/v) with carbohydrate at the ratio of protein to carbohydrate of 0.3:0.7. The total solid concentration of feed prior to spray-drying was 20 %. The suspensions were fed at 4.0±0.5 mL min^{-1} and dried using

inlet air of 130 °C and outlet air of 100 °C, with the flow rate of drying air around 600 Nm^3 min^{-1} (Mini Spray Dryer B-19-; Buchi, Flawil, Switzerland). Each spray-dried sample was prepared in duplicate, sealed in a polyethylene laminated aluminium foil bag and stored at -20 ℃ prior to analysis. They were designated as WPC-TH, WPC-MD, WPH-TH and WPH-TH, respectively.

2.3 Conformational changes of proteins determined by fluorescence spectroscopy

The intrinsic fluorescence of whey proteins was evaluated to determine the influences of carbohydrate excipients on conformational changes of proteins after tryptic hydrolysis and spray-drying. Steady-state fluorescence spectroscopy is commonly used to evaluate protein conformational changes based on the exposure of aromatic amino acid residues contributing fluorescence characteristics of proteins, i.e. tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues as fluorophores (Rudra, Dasmandal, Patra, & Mahapatra, 2018). Fluorescence measurement was carried out using a multifunctional microplate reader (Tecan, Infinite®M200 PRO, Grödig, Austria). Emission spectra were recorded from 290 to 510 nm with an excitation wavelength of 280 nm as described by Shpigelman, Israeli, & Livney (2010).

Quenching of Trp and Tyr fluorescence in WPC-TH, WPC-MD, WPH-TH and WPH-MD by cyanidin-3-glucoside (C3G) quencher was used to monitor the fluorophore-site accessible to the ligand/quencher of dairy proteins (He et al., 2018). This is because the fluorescence properties of proteins are quite sensitive to microenvironment (Rudra et al., 2018). The carbohydrate excipients of different MWs used during spray-drying may alter solvent properties such as polarity and osmotic pressure, leading to the changes in accessibility of Trp and Tyr fluorophores that can be accessed by C3G quencher.

Cyanidin-3-glucoside solution (0, 25, 30, 40 or 50 μM in 30 mM phosphate buffer, pH 2.5) was added to whey protein and hydrolysate solutions (1 mg mL^{-1} protein in 30 mM phosphate buffer, pH 2.5). After addition, the mixture was thoroughly mixed and heated to 35°C, 45°C or 65°C for 30 min and cooled to 27 °C (room temperature). The emission spectra of the mixed suspensions were recorded from 290 to 510 nm using an excitation wavelength of 280 nm in a microplate reader (Tecan, Infinite®M200 PRO, Grödig, Austria).

Normally fluorescence quenching was described by the Stern – Volmer equation (Eq 1):

$$\frac{F_0}{F} = 1 + K_{SV} * [Q] \qquad \text{Eq 1}$$

where F_0 and F are fluorescence intensity in the absence and presence of C3G quencher, K_{sv} is Stern – Volmer quenching constant and $[Q]$ is the concentration of C3G. However, the linear Stern – Volmer plot (an indicative of a single class of fluorophores) was not achieved in this study (results not shown). The modified Stern – Volmer equation (Eq 2) was used instead. The deviation from linearity of Stern – Volmer equation Eq 1 suggested the existence of a population of inaccessible fluorophores (Lakowicz, 2006; Shpigelman et al., 2010).

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a * K_{SVa} * [Q]} + \frac{1}{f_a} \qquad \text{Eq 2}$$

where F and F_0 are fluorescence intensities of protein solution in the presence and absence of C3G quencher, f_a is the fraction of fluorophore-site accessible to C3G, $[Q]$ is the C3G concentration and K_{SVa} is the Stern – Volmer quenching constant of the accessible fraction. The calculated f_a and K_{SVa} were reported.

2.4 Effects of storage condition and carbohydrate excipients on physicochemical properties of reconstituted powder

The spray-dried powder was kept in a desiccator containing P_2O_5 powder for 7 days to adjust the moisture content close to zero prior to storage at different storage conditions at 11% and 24% RH over the saturated solution of LiCl (11%RH) and CH_3COOK (24%RH). The storage temperature of 35°C in an incubator (Memmert GmbH, Schwabach, Germany) was used to imitate the common temperature in the tropic and the powder was kept for 5 days.

2.4.1 Two-dimensional gel electrophoresis

The MWs and isoelectric pH (pI) profiles of proteins and peptides in reconstituted WPC-TH, WPC-MD, WPH-TH and WPH-MD before and after storage were evaluated using the method described by Friedman, Hoving & Westermeier (2009). Immobilized pH gradient (IPG) strips pH 3-10 with a length of 7 cm were used in the separation of the first dimension. The powder (2 mg protein mL^{-1}) was solubilized in 125 µL rehydration solution (8 M urea, 2% (w/w) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, (CHAPS), 0.002% (w/v) bromophenol blue, 60 mM dithiothreitol (DTT) and 0.5% (w/v) immobilized dried pH gradient buffer, pH 3-10). The IPG strips were rehydrated in immobiline drystrip reswelling tray (Amersham Biosciences AB, Uppsala, Sweden) with rehydration solution of 125 µL per strip, and covered with dry strip cover fluid to prevent urea crystallization during the rehydration process. The strip was rehydrated at room temperature for 12 hrs.

The first dimension isoelectric focusing (ETTAN IPG PHOR 2, Amersham Biosciences AB, Uppsala, Sweden) was run at 20 °C with total voltage-hour (V.hr) of approximately 6.5 V.hr according to the program: (1) step and held at 300 V, for 0.3 kV.hr; (2) gradient at 1000 V for 0.3 kV.hr; (3) gradient at 5000 V for 4 kV.hr and (4) step and hold at 5000 V for 3 kV.hr.

After the completion of the first dimension, IPG strip was equilibrated in a 5 mL equilibration solution (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue with 1% (w/v) DTT). The solution was shaken gently for 15 min at room temperature. Then equilibration solution with DTT was removed and substituted with 5 mL of equilibration solution with 2.5% (w/v) iodoacetamide (IAA). The solution was gently shaken for 15 min. Afterwards, the IPG strip was rinsed with SDS electrophoresis buffer (25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS) and subjected to the second dimension, SDS-PAGE.

SDS-PAGE was carried out on 12.5% running gel (41.7% (w/v) acrylamide/Bis, 25% (w/v) 1.5M Tris-HCl pH 8.8, 1% (w/v) of 10% SDS, 0.5% (w/v) of 10% APS, 0.33% (w/v) TEMED and 31.8% double deionized water), polymerized for at least 1 hr before running. The IPG strip was placed onto the gel. The 8 µL of MW marker (PlusOne, Amersham Biosciences AB, Uppsala, Sweden) was applied on the 0.5x1 cm^2 filtered paper and dried at room temperature for 5 min. The filtered paper containing molecular weight marker was placed next to the acidic end of IPG strip. The IPG strip and MW markers was overlayed with 1.5% agarose (1.5% (w/v) agarose, 0.002% (w/v) bromophenol blue) (80°C). The SDS-PAGE program was 10 mA/strip, for 15 min followed by 20 mA per strip for 1.3 hrs. Gel was stained using Comassie brilliant blue staining solution (40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Comassie brilliant blue R-250) for 30 min then destained (10% (v/v methanol), 7.5% (v/v) acetic acid). The pH scale in the gel was calculated using guidelines to choose gel length, pH gradient and estimation of pI value (GE lifesciences, 2012).

2.4.2 Oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$)

Powder before and after storage was reconstituted in 0.1M phosphate buffer, pH 8.0 to obtain 1.3 % protein (w/v) and evaluated for $ORAC_{FL}$ assay determined using method described by Prior et al. (2003). Briefly, the $ORAC_{FL}$ assays were carried out on a multifunctional microplate reader (Tecan). The reaction was carried out at 37°C in 75 mM phosphate buffer, pH 7.4 and final reaction mixture was 200 µL. Antioxidant (20 µL; Trolox (10 90 µM) or sample) and fluorescein (120 µL; 700 nM, final concentration) were loaded to black 96-well microplates. The plate reader (Tecan) was programmed to record the fluorescence of fluorescein on every cycle. There were 60 cycles. Each cycle was run for 2 min and 39 s with no time lapse between cycles. During cycle 4, plate was

removed and 60 μL of 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) was manually injected into the respective wells to give a final AAPH concentration of 12 mM. The plate contents were mixed by shaking for 8 s. The temperature of the incubator was set to 37°C, and a fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. The final $ORAC_{FL}$ values were calculated by using a linear equation between the Trolox standards or sample concentration and net areas under the fluorescein decay curves. The area under curve (AUC) was calculated as shown in Eq 3:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 +, ..., + f_i/f_4) \times CT \quad \text{Eq 3}$$

where f_4 was the initial fluorescence reading at cycle 4, f_i was fluorescence reading at cycle *i*, and CT was cycle time in minutes. The net AUC was obtained by subtracting the AUC of the blank from that of a sample. The $ORAC_{FL}$ values of samples were then reported as μmol trolox equivalent per mg of protein (μmol TE mg^{-1} protein).

2.4.3 Thiol group content

Thiol group content in reconstituted samples was determined using the method described by Beveridge, Toma, & Nakai (1974). The 0.5 mL of reconstituted protein (1.3 % protein w/v) in Tris-glycine buffer, pH 8.0 was added into 2.5 mL of 8 M urea in Tris-glycine buffer. Ellman's reagent (4 mg of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 1 mL of Tris-glycine buffer) was added to the solution and mixed thoroughly. The solution was let to stand at room temperature for 15 min and measured for the absorbance at 412 nm using multifunctional microplate reader (Tecan). The thiol contents were calculated according to the Eq 4 described below and expressed as μM g^{-1} protein.

$$SH\ content\ (\mu M / g\ protein) = \frac{73.54 \times Abs_{412} \times 6.04}{Sample\ concentration\ (mg\ mL^{-1})} \quad \text{Eq 4}$$

2.4.4 Moisture content

The moisture content of spray-dried powder before and after storage was assessed by Karl Fisher titration (Karl Fischer Titration; Metrohm AG, Herisau, Switzerland). Sample mass was around 0.004 – 0.006 g. Dry methanol (Hydranal®, Riedel-de Haën, Seelze, Germany) mixed with chloroform in the ratio of 1:1 was used as the titration reagent.

2.4.5 Glass transition temperature

Spray-dried powder (3-10 mg) was transferred to a stainless pan and analyzed for glass transition temperature (Tg) using a differential scanning calorimetry (DSC, Pyris1, Perkin-Elmer, Norwalk, CT, USA). The sample was scanned at 5 $^{\circ}$C min^{-1} from – 40 $^{\circ}$C to 130 $^{\circ}$C using method described by Jouppila & Roos (1994).

2.5 Statistical analysis

Experiments were carried out in two separate trials of spray – drying; each trial was analyzed in triplicate. The data were analyzed by analysis of variance (ANOVA) with significance at $P < 0.05$. Significant differences among mean values were determined by Tukey's test. All statistical analyses were performed using SPSS software Version 12 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Conformational transition of whey proteins after tryptic hydrolysis and spray drying

Reconstituted protein and hydrolysate showed some conformational transition of hydrophobic residues, observed as lower intrinsic fluorescence intensity in the hydrolysate (WPH-TH) compared to protein (WPC-TH) **(Fig. 19a).** The intrinsic fluorescence is mainly dominated by the emission of Trp and Tyr residues under the excitation wavelength of 280 nm (He et al*.,* 2018). The lower fluorescence intensity of Trp and Tyr in WPH despite low degree of hydrolysis may be due to the low pH during trypsin inactivation prior to spray-drying. The acidification of WPH may perturb the microenvironment of the aromatic amino acid residues in WPH, leading to the lower exposure of Trp and Tyr.

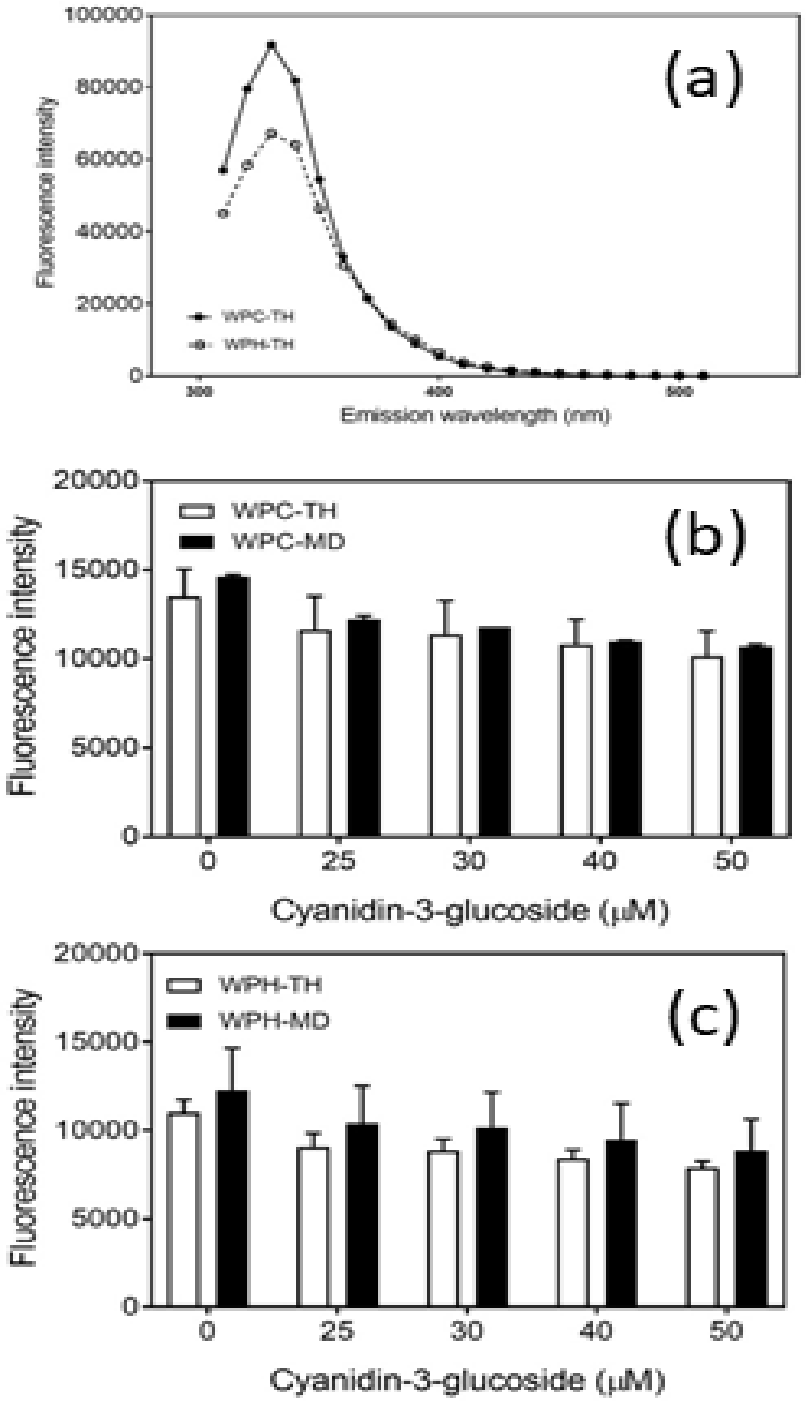

Fig. 19 Effect of trehalose (TH) and maltodextrin (MD) on fluorescence intensity of 1 mg mL^{-1} protein suspensions containing reconstituted whey protein concentrate (WPC) or whey protein hydrolysate (WPH): (a) emission spectra of protein suspension at excitation wavelength 280 nm); (b) fluorescent quenching of reconstituted WPC emitted at 332 nm by cyanidin-3-O-glucoside at 35 °C; (c) fluorescent quenching of reconstituted WPH emitted at 332 nm by cyanidin-3-O-glucoside at 35 °C

Fluorescence quenching of fluorophore residue by C3G quencher also indicated that increasing C3G concentration lowered the fluorescence intensity of proteins in both WPC **(Fig. 19b)** and WPH **(Fig. 19c)** regardless of carbohydrate excipients. However, to distinguish between non-binding-induced (dynamic) quenching from binding (static) quenching of protein fluorophore with C3G quencher, the reaction temperatures were increased from 35 °C to 45 °C and 65 °C, which were lower than the denaturation temperatures of major whey protein β-Lg. The α-La, however, could adopt molten globule state at acidic pH at elevated temperature of 65 °C. Irreversible protein unfolding during fluorescence quenching characterization resulted from covalent crosslinks between protein-protein and protein-polyphenol was also minimized at acidic pH.

Fluorescence quenching analysis revealed that the carbohydrate excipients significantly influenced fluorophore accessible site to C3G (f_a) of WPC and WPH, particularly at 35 and 45 °C **(Table 9).** The efficiency of quenching or Stern – Volmer quenching constant of the accessible fraction (K_{SVa}) of protein in WPC-TH, WPC-MD, WPH-TH and WPH-MD were not significantly different ($P \geq 0.05$). The higher *fa* values of reconstituted WPC and WPH when MD was used as excipient compared to those embedded in TH indicated that the presence of MD partially exposed accessible site on proteins (Trp and Tyr) more than did TH.

Table 9 Effect of excipient types on fluorophore-site accessible to C3G fraction (f_a) and Stern – Volmer quenching constant (K_{SVa}) at different temperature

Sample	f_a			K_{SVa} [10^5 M^{-1}]		
	35°C	45°C	65°C	35°C	45°C	65°C
WPC-TH	0.366±0.059^b	0.325±0.153^b	0.520±0.144^a	3.182±1.786^a	3.121±1.832^a	1.104±0.498^a
WPC-MD	0.376±0.033^a	0.365±0.020^a	0.389±0.004^a	3.408±0.948^a	3.238±0.659^a	2.632±0.108^a
TWP-TH	0.358±0.046^b	0.370±0.006^b	0.367±0.000^b	4.149±1.357^a	3.408±0.236^a	3.480±0.162^a
TWP-MD	0.395±0.001^a	0.433±0.033^a	0.394±0.006^a	2.509±0.171^a	2.021±0.359^a	2.281±0.049^a

Data represent mean values ± standard deviations of two replicates. Measured values followed by the same superscript letter in a column are not statistically different ($P \geq 0.05$).

In this study, the modified Stern-Volmer equation was used due to curvature appearance of Stern-Volmer plot instead of linearity (result not shown), indicating the actions of both static and dynamic quenching. However, the increase in reaction temperatures from 35 to 65 °C did not significantly change the Stern-Volmer quenching constant of accessible fraction K_{SVa}. This indicated that dynamic quenching was predominant; and the quenching was from the excited state of fluorophore rather than the formation of ground state bound complex (Hu, Liu, & Xiao, 2009; Lakowicz, 2006). Although the complexation between whey proteins/peptides and C3G via non-covalent binding did not occur below 65 °C, the results suggested that MD and TH excipients could differently perturb the microenvironment surrounding the aromatic amino acid residues and exposed them to the different degree.

3.2 Effects of storage condition carbohydrate excipients on physicochemical changes of reconstituted powder

The 2-D gel electrophoresis illustrates the major whey proteins α-La and β-Lg in WPC (Fig. 20) and WPH (Fig. 21), observed around 14 and 18 kDa and pI around 5.2 respectively, regardless of excipient types. The WPC (Fig. 20a and Fig. 20b) also contained proteins having MW close to 70 kDa and pI around 5.5. Hydrolysis by trypsin at 37 °C for 1 h partially hydrolyzed proteins in WPC, particularly those that have MWs above 17 kDa (Fig.21). The use of either TH or MD during drying process did not have the effect on the types of proteins and peptides present prior to storage at 24% RH, 35 °C for 5 days (Fig.21).

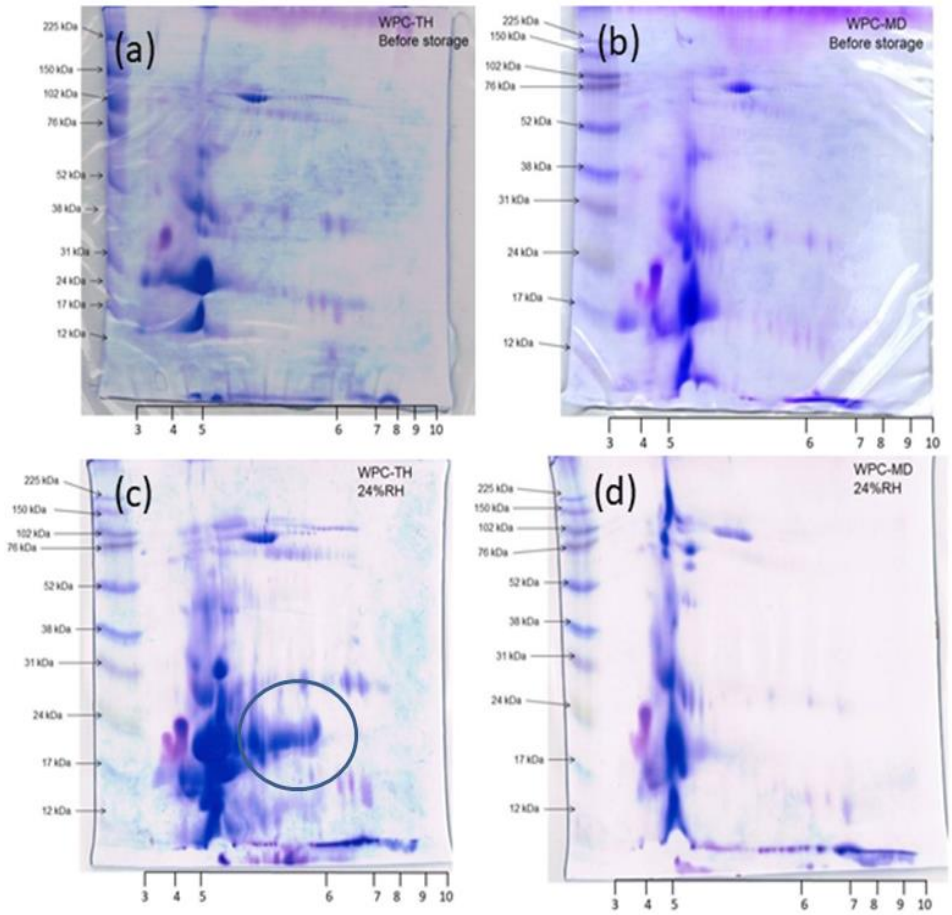

Fig. 20 Two-dimensional gel electrophoresis of WPC spray-dried with trehalose (TH) or maltodextrin (MD) before (a,b) and after (c,d) storage at 24% RH, 35 °C for 5 days.

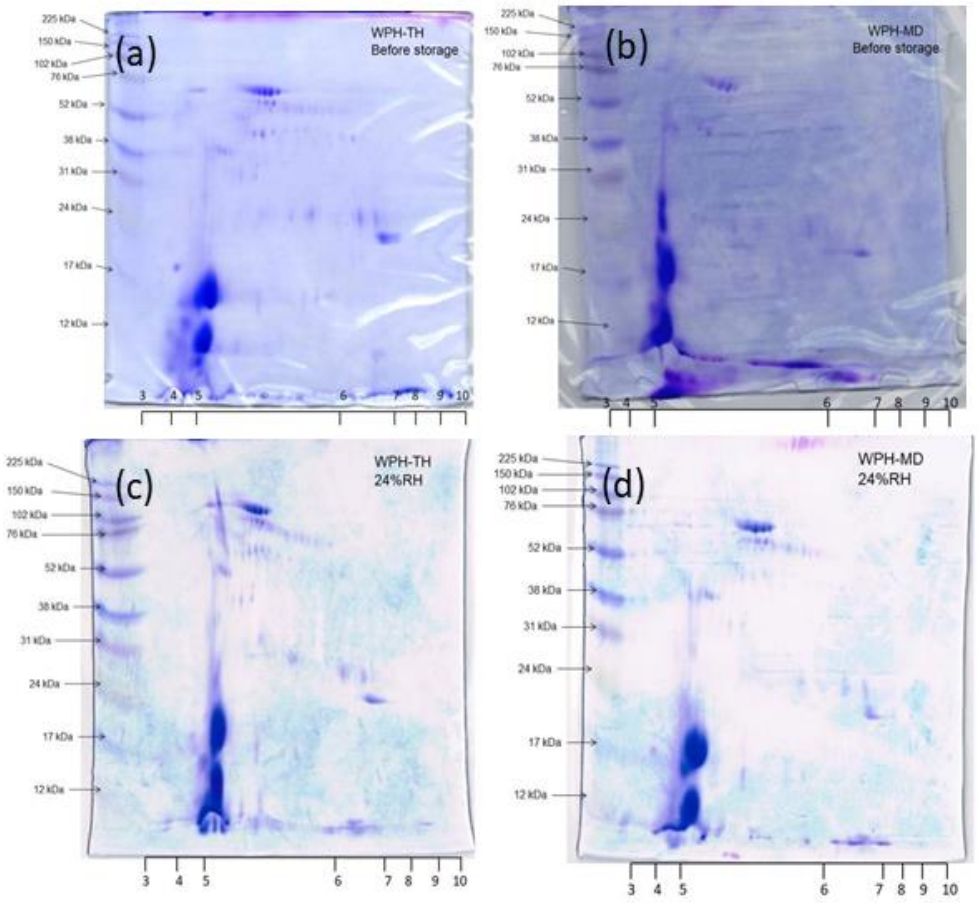

Fig. 21 Two-dimensional gel electrophoresis of WPH spray-dried with trehalose (TH) or maltodextrin (MD) before (a,b) and after (c,d) storage at 24% RH, 35 °C for 5 days.

Storage at 24% RH, however, induced aggregation of proteins in WPC-TH (Fig. 20c) to high extent. Such aggregation of proteins was likely induced by sorbed water shown as an increase in moisture content (Fig. 22a) and lower Tg (Fig. 22b). Aggregation of proteins in WPC-TH during storage resulted in the formation of new protein species having MWs around 12-225 kDa and pIs around 5-10 (Fig. 20c). The aggregation of proteins in WPC-MD after storage at 24 %RH at 35 °C for 5 days, however, was not apparent as those spray-dried in the presence of TH excipient (Fig. 20d). In contrast to WPC, the WPH was not subjected to aggregation after storage regardless of carbohydrate excipients (Figs. 21c, 21d)

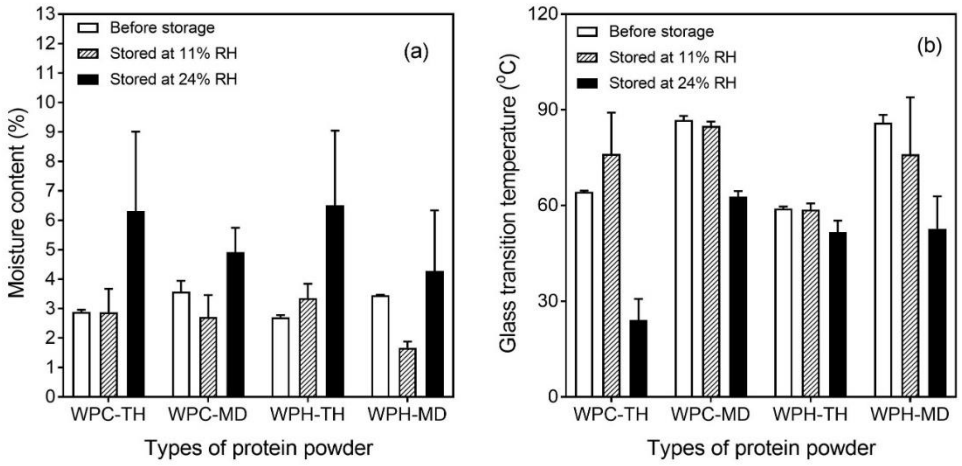

Fig. 22 Effect of trehalose (TH) and maltodextrin (MD) on (a) moisture content and (b) glass transition temperature of WPC and WPH stored at 11 and 24% RH at 35 °C for 5 days. Bars represent standard deviations.

Solid state deterioration caused by sorbed water during storage involved non-covalent and covalent modification of protein, peptide and amino acid (Costantino et al., 1994; 1995; Liu et al., 1991; Schwendeman et al., 1995; Lai & Topp, 1999). Moisture-induced aggregation of proteins is usually initiated by surface induced aggregation *via* van der Waals forces, hydrophobic interactions, electrostatic interactions and H-bonds (Wang, 2005). These non-covalent forces could be dissociated by 2-D gel electrophoresis rehydration solution in the presence of urea, CHAPS and DTT (Friedman, Hoving & Westermeier, 2009). The aggregation of proteins in WPH-TH induced by sorbed water after storage at 24 %RH, 35 °C for 5 days was likely driven by the formation of covalent non-disulfide bonds that could not be dissociated by the rehydration solution (Lai & Topp, 1999). However, such aggregation was not found in reconstituted WPC-MD after storage.

It is possible that small MW excipient like TH resulted in different conformation transition and alignment of proteins compared to those in the presence of MD after mixing with protein suspensions and spray-drying. Different alignments of protein governed by different carbohydrate excipient TH or MD were shown, in part, as different fluorophore-site accessible to C3G fraction (f_a) of WPC (Table 9). This could lead to different reactive groups responsible for moisture-induced aggregation and polymerization during storage at 24% RH. Osmotic effect of TH could induce depletion flocculation of proteins at the faster rate compared to MD (McClements, 2000).

However, the partially hydrolysed proteins in WPH were not prone to moisture-induced aggregation after storage at 24 %RH (Fig. 21), regardless of carbohydrate excipient. Unlike the influences of short chain peptide and amino acid influences on WPH reported by Zhou et al. (2014); of which the

powder was prepared in the absence of excipient. This may be due to different degree of hydrolysis and the use of glassy carbohydrate matrix in the protein formulation reported in this study.

Table 10 shows that the thiol group content of WPC and WPH remained unchanged before and after storage, indicating that there was no increase in disulfide bond formation. Nonetheless, the $ORAC_{FL}$ antioxidant capacity of WPH drastically reduced after storage at 24 % RH. The reduction of $ORAC_{FL}$ occurred to high extent in WPH than in WPC, regardless of carbohydrate excipient. The sorbed water at 24% RH storage condition may influence further interactions of reactive groups of proteins and peptides responsible for oxygen radical scavenging ability via hydrogen atom transfer mechanisms.

Table 10 Effect of excipient on thiol group content and oxygen radical antioxidant before and after storage at 35 °C for 5 days

Sample	SH group content (μM/g protein)			$ORAC_{FL}$ (μg Trolox equivalent/mg protein)		
	Before storage	Storage at 11% RH	Storage at 24% RH	Before storage	Storage at 11% RH	Storage at 24% RH
WPC-TH	17.0±0.1[a]	15.6±0.4[a]	16.0±0.5[a]	0.28±0.03[b]	0.14±0.01[b]	0.07±0.01[a]
WPC-MD	16.9±0.3[a]	15.3±0.5[a]	16.7±0.8[a]	0.23±0.02[b]	0.17±0.01[b]	0.09±0.04[a]
TWP-TH	16.1±0.7[a]	14.7±1.7[a]	17.1±0.2[a]	0.66±0.19[a]	0.88±0.36[a]	0.17±0.06[a]
TWP-MD	17.0±1.2[a]	14.9±0.1[a]	16.3±0.5[a]	0.49±0.01[ab]	0.41±0.02[ab]	0.10±0.00[a]

Data represent mean values ± standard deviations of two replicates. Measured values followed by the same superscript letter in a column are not statistically different ($P \geq 0.05$).

This study shows the influences of MD as effective carbohydrate excipient for WPC and WPH in preventing moisture-induced aggregation at 35 °C, which is typical temperature in the tropical region. The steric hindrance of starch hydrolysate in MD may help reduce mobility of proteins and peptides during storage at such temperature. Nonetheless, choosing a suitable carbohydrate excipient for stabilizing the bioactive dairy protein hydrolysate powder requires further investigation on the protein formulation and storage condition.

4. Conclusions

Carbohydrate excipient can play significant roles on conformational changes of whey proteins before and after storage the powder at 35 °C. The high MW maltodextrin may have advantage over small MW sugar TH in preventing moisture-induced aggregation of proteins in dried state.

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4.2 Self-assembly of peptide-carbohydrate aggregates during *in vitro* gastrointestinal digestion of mungbean protein hydrolysate and mungbean protein hydrolysate-asiatic Acid

ABSTRACT

Association of proteins and peptides in the digesta during *in vitro* gastrointestinal digestion resulted in the formation of large aggregates having different length-scale size despite the hydrolysis of proteins and peptides to smaller molecular weight (MW) compounds including amino acids. This study explored the influence of amphiphilic pentacyclic triterpene anticancer asiatic acid (AA) on the fates of mungbean protein hydrolysate (MPH) during *in vitro* gastric and small intestinal digestion in the presence of maltodextrin and starch. The low pH during gastric digestion induced aggregation of MPH and MPH-AA from 300 nm to micrometer size range (> 1000 nm) although the MPH was hydrolysed by pepsin during stomach phase. The presence of amphiphilic AA in MPH-AA, however, helped retain small length-scale size of the digested MPH-AA aggregates during digestion during intestinal phase. The digested MPH in the absence of AA was prone to aggregation compared to the digested MPH-AA during gastrointestinal hydrolysis due to the difference in charge and surface properties. The self-assembly of digested aggregates in the digesta was pronounced when the high MW carbohydrates were also hydrolysed to dextrin during *in vitro* intestinal digestion. This study has highlighted the dynamic of self-assembly of peptides during *in vitro* gastrointestinal digestion.

Introduction

Proteins, peptides and amphiphilic molecules having bioactivities such as antioxidative activities can further aggregatein aqueous phase with various properties due to hydrophobic moieties in the molecules. Food preparation conditions such as pH, ionic strength, concentration, enzymatic hydrolysis, drying method, the presence of surface active and surface inactive agents prior to consumption also influenced the aggregate size and biological activities of proteins and peptides.[1-4]

Mungbean are an important source of pulse protein and starch in Asia. In Thailand, protein is a by-product from mungbean starch and vermicelli production and is sold as animal feed. Mungbean protein isolate could perform many desirable functions in processed foods, such as foaming, emulsifying, gelling and water absorption.[5-8] However, research on aggregation characteristics of reconstituted mungbean protein hydrolysate (MPH) during *in vitro* gastrointestinal digestion is limited.

The amphiphilic molecule asiatic acid (AA) is a pentacyclictriterpene found in *Centella asiatica* Linn. or bua-bok exhibits anticancer activity.[9] AA has low hydrophile-lipophile balance (HLB), thus low solubility in water or phosphate buffered saline.[10-11] It usually forms micelle in aqueous solution and the size could be too large to be taken up at the cellular level. We have previously demonstrated that mungbean protein hydrolysate (MPH) obtained from tryptic hydrolysis for 30 min could be used as effective carrier for AA that enhanced AA efficacy in human colon adenocarcinoma SW620, human hepatoblastoma HepG2, and diploid monkey kidney cells (Vero).[12] However, for oral consumption of AA encapsulated in mungbean peptide nanocluster, further changes of the peptide fractions may occure particularly when carbohydrate excipients are also present.

The fates of protein- or peptide-based nano-carrier under GI conditions and in the presence of carbohydrates thus deserve further investigation. The presence of viscosity-promoting carbohydrates such as starch and maltodextrin influence not only physical characteristics and flow properties, but also, by mixing of digesta, the absorption of bioactive compounds within the lumen of the intestine.[13-14] Particles having size below 300 nm could be passively absorbed at cellular levels.[15-19]

This research hypothesized that physiological conditions in the GI tract (pH alterations, enzymatic hydrolysis and time duration) could influence aggregation nature of reconstituted spray-dried MPH and MPH-AA after ingestion and subsequent particle size distribution prior to cellular uptake. The objective of this research was to elucidate the influences of high MW carbohydrate excipients on the aggregation characteristic of MPH and MPH-AA complexes during *in vitro* digestion. Maltodextrin-starch (MS) mixture excipients encapsulating MPH and AA-MPH were used as carbohydrate model materials.

2. Materials and Methods

2.1 Materials

De-hulled mungbean grains (Raithip, Bangkok, Thailand) were purchased from a local supermarket.Trypsin (EC 3.4.21.4; 10,000 BAEE U mg-1), asiatic acid (AA), and fluorescein (Na salt) werepurchased from Sigma-Aldrich (St. Louis, MO).2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH), and1-anilino-8-naphthalenesulfonate (ANS) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium dihydrogenorthophosphate, disodium hydrogen orthophosphate dihydrate, and sodium chloride werepurchased from Ajax Finechem (Seven Hills, NSW,Australia). Cassava maltodextrinwithdextrose equivalent (DE) of 18 (Neo-Maldex®) was purchased from Neotech Food Co.(Ratchaburi, Thailand).

2.2 Preparation of mungbean protein concentrate (MPC), mungbean protein hydrlysate (MPH) and spray-dried MPH and MPH-AA in high MW carbohydrate excipient

Mungbean protein concentrate (MPC) and tryptic hydrolysed mungbean protein hydrlysate (MPH) were prepared from de-hulled mungbean flour using methods previously described.[20] MPH and MPH-AA in maltodextrin: starch (9:1) excipient were chosen for spray-drying by a minispray-dryer (model B-190, Buchi, Flawil, Switzerland), using air inlet temperature of 130 °C, outlet temperature around 90 °C and flow rate 3-4 mL/min. Spray-dried powders were kept in desiccator with P_2O_5 for 1 week, packed in aluminiumfoil PE laminated bag and kept in a -20 °C freezer. Before analysis, the powder was reconstituted by dispersing in distilled water unless stated otherwise.

2.3 Effect of *in vitro* digestion on aggregation characteristic of MPH and MPH-AA in maltodextrin-starch excipient

Freshly prepared MPH (total solid of 4 %) and reconstituted MPH and MPH-AA co-dried in mixture of maltodextrin-starch excipients reconstituted in double deionized water to obtain total solid of 11.72-11.94 % were investigated. They were adjusted to pH 2.1 using 5 M HCl and digested by pepsin for 1 h at 37 °C (protein to enzyme ratio of 25: 1) during gastric phase. The digesta were adjusted to pH 5.3 using 0.9 M $NaHCO_3$ and then adjusted to pH 7.5 using 5 M NaOH. Pancreatin was added to the digesta by using protein to enzyme ratio of 35: 1 and digested for 1 h at 37 °C to imitate intestinal phase.[21] Samples from each step were frozen in liquid nitrogen and kept in a -80 °C freezer until analysed. The particle size distribution and molecular size distribution of the digesta were analysed using methods described below.

2.3.1 Length-scale size distribution

Size distribution of digesta from freshly prepared MPH suspension and reconstituted powder were measured by diluting the mixture solution using filtered (0.22 μm; Millipore, Billerica, MA, USA) deionized water to obtain a final protein concentration of 0.14% w/v. They were analyzed by Zetasizer (model ZS90, Malvern Instruments, Malvern, Worcestershire, UK).

2.3.2 High performance size exclusion chromatography with refractive index detection *(HPSEC-RI)*

Four hundred microliters of freshly prepared MPH and reconstituted MPH-AA in mixture of maltodextrin-starch carbohydrate excipients were dispersed in filtered double deionized (DI) water. All digesta were diluted in 1.6 mL of filtered double DI water (0.22 μm, Millipore, Billerica, MA,

USA)with 0.02% $NaNO_3$ and filtered through 0.45 µm of nylon filter (Daigger, Vernon Hills, Il, USA). The final concentrations of samples are 2.04-2.34 % w/v, containing 0.6-0.7 % protein (w/v), 0.036-0.044 % AA (w/v) and 1.4-1.6 % carbohydrates (maltodextrin-starch mix) (w/v). Two hundred microliters of samples were injected into a high performance size exclusion chromatography-refractive index (HPSEC-RI) Variance system: a pump (Varian 9012, Varian Associates, Walnut Creek, CA, USA), a syringe sample loading injector (model 7125, Rheodyne, Oak Harbor, WA, USA) with 200 µL sample loop, and a refractive index detector (Varian star 9040, Varian Associates, Walnut Creek, CA, USA). The systems were connected with size exclusion column: 1st column Superdex 30 and 2nd column Superdex 200 (Amersham Biosciences, Piscataway, NJ, USA). The running conditions using flow rate of 0.4 mL/min at 25 °C and 0.45 µm filtered double DI water was used as mobile phase. Experimental data were collected using Varian's Star Chromatography Workstation (version 4.51, Varian Associates, Walnut Creek, CA, USA).

3. Results & Discussion

3.1 Length-scale size distribution of MPH and spray-dried MPH and MPH-AA in high MW carbohydrate excipient

After reconstitution in double DI water, the Z-average sizes of all samples were around 200 nm ($P \geq 0.05$) with monomodal distribution (**Table 11).** This result suggests that spray-drying of MPH and MPH-AA in carbohydrate excipients retained small size of particle after reconstitution. Spray-drying is the short time process, maltodextrin and starch are good glass forming matrices. They can form amorphous structure and immobilize MPH and MPH-AA in their glassy matrices.

Table 11 Size distribution of reconstituted spray dried MPH and MPH-AA in maltodextrin-starch excipient. The suspensions contained 0.40 % total solid and protein: AA: carbohydrate ratio of 1: 0.072: 2.34.

Reconstituted spray dried powder	Z-average size (nm) [ns]
MPH-MS	208 ± 12
MPH-AA-MS	226 ± 15

[ns] Indicates no significant difference ($P \geq 0.05$).

3.2 Effect of *in vitro* digestion on aggregation characteristic of MPH and MPH-AA in maltodextrin-starch excipient

3.2.1Length-scale size distribution

The size distribution between freshly prepared MPH and all reconstituted mixtures during simulated gastrointestinal digestion were compared **(Fig. 23).** In the absence of carbohydrate, freshly prepared MPH consisted of small and large aggregated peptides, the size of which ranged from 100 nm to 4 μm respectively. In contrast, all reconstituted hydrolysate had particle size around 300 nm and showed monomodal distribution. This result suggested that spray-drying of MPH and MPH-AA in carbohydrate excipients retained small size of particle after reconstitution. Spray-drying MPH and MPH-AA in maltodextrin-starch excipient was effective to control particle size to submicron size. However, aggregation of MPH and MPH-AA dramatically occurred after adjusting pH to 2.1 and peptic digestion during gastric phase. The sizes of aggregate were larger than 1000 nm. This result suggested that acidic pH is the driving force for re-aggregation of MPH and MPH-AA during gastric digestion despite that all peptides were further hydrolysed by pepsin. After pH adjustment to 7.5, re-aggregation of MPH and MPH-AA occurred again. However, the aggregates were smaller within the submicron size after being digested under small intestinal condition while the large-sized aggregates still existed.

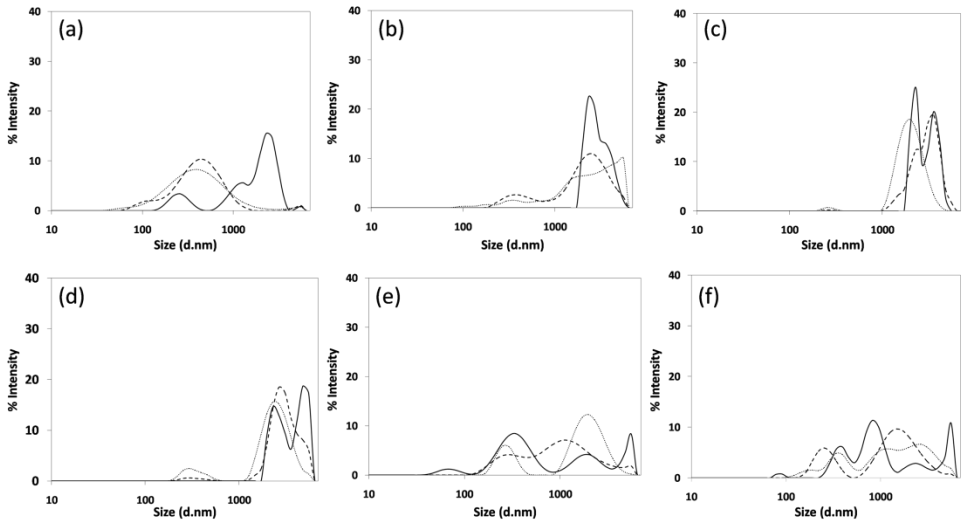

Fig. 23 Size distribution of freshly prepared MPH (———), MPH in maltodextrin - starch excipient or MPH-MS (·····) and MPH-AA in maltodextrin - starch excipient or MPH-MS-AA (- - - - -) during gastrointestinal digestion: (a) after reconstituted, (b) adjusted pH to 2.1, (c) hydrolysed by pepsin 60 min, (d) adjusted pH to 5.3, (e) adjusted pH to 7.5 and (f) hydrolysed by pancreatin 60 min.

3.2.2 High performance size exclusion chromatography with refractive index detection

Fig. 24 shows HPSEC-RI profile of digesta in gastric phases. The MWs of the MPH and MPH-AA were lowered due to further hydrolysis of peptides. However, the presence of starch and maltodextrin promoted depletion flocculation and induced re-aggregation of the MPH to a higher MW. After digestion by pancreatin, which is composed of α-amylase, trypsin and lipase, maltodextrin and starch were hydrolysed into maltose and glucose. Depletion flocculation still occured and induced re-aggregation of the MPH to the higher MW. However, in the presence of AA, MPH-AA co-aggregates were quite stable to the depletion effect from small MW carbohydrates and sugars. This confirmed that AA, which is amphiphilic molecule having molecular structure close to bile acid, helped stabilize MPH aggregation in the presence of small MWsaccharides.HPSEC-RI profile of MPH during the gastric phase confirmed that the aggregation profile of MPH was changed by gastric digestion by pepsin for 60 min (Fig 2a).

During the intestinal phase by pancreatin for 60 min, the amount of small MW peptides were increased (**Fig 24b**). This suggested that the peptic hydrolysed MPH was further hydrolysed by trypsin during the *in vitro* digestion. Physiological conditions in the GI tract influenced the aggregation nature of reconstituted MPH and MPH-AA complexes during in vitro gastrointestinaldigestion. Altering pH during digestion was the major driver controlling the size of the hydrolysed aggregates. Acidic pH during gastric phase enhanced re-aggregation to micrometer length-scale size of particle. Under small intestinal conditions, however, where maltodextrin and starch were hydrolysed to smaller saccharides, the re-aggregation also occurred due to depletion flocculation induced by increased osmotic pressure of sugars. Such re-aggregation could be detrimental to cellular uptake via passive diffusion since the majority of the aggregates were larger than 300 nm. Nonetheless, the presence of AA helped retaining the 300 nm aggregates more effectively than in the absence of AA.

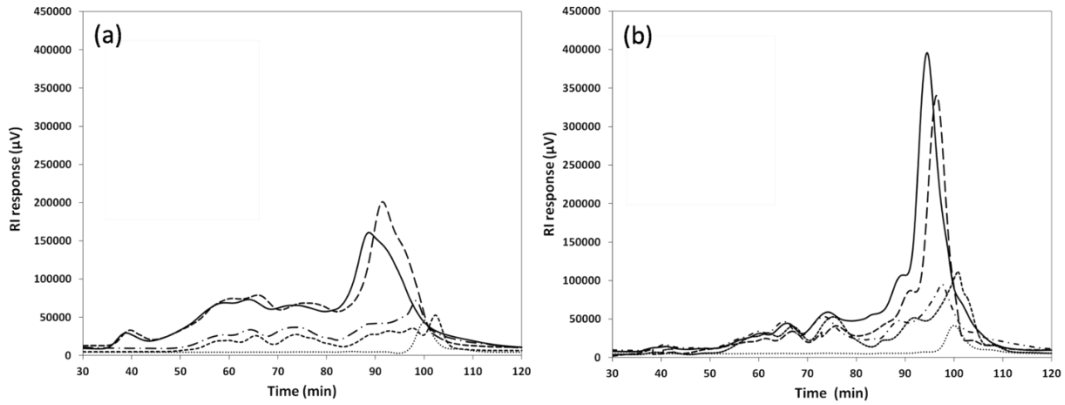

Fig. 24 HPSEC-RI profile of MPH (- - - - -), MPH-MS (———), MPH-MS-AA (— — —), MPH-AA (- · - · - ·), AA (·····) after digestion by (a) pepsin for 60 min and (b) pancreatin 60 min. The suspensions contained 2.04-2.34 % total solid in filtered double deionized water and protein: AA: carbohydrate ratio of 1: 0.072: 2.34.

4. Conclusion

The insights in understanding the effects of amphiphilic pentacyclic molecule like AA, carbohydrate excipient, changes of pH and physiological condition during digestion may help to better controlling functions of peptide-based encapsulation in amorphous carbohydrate excipient for dry products and their functions in the GI-tract and explain differences in bioavailability of bioactive compounds.

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บทที่ 5 ข้อเสนอแนะ

แม้ว่างานวิจัยนี้จะเป็นงานวิจัยพื้นฐาน แต่องค์ความรู้จากงานวิจัยนี้สามารถนำไปใช้ในอุตสาหกรรมอาหารได้โดยตรง เนื่องจากการใช้โปรตีนไฮโดรไลเสทลดการเกิดสีน้ำตาลจากปฏิกิริยาเมลลาร์ดในอาหารที่ผ่านความร้อนแทนการใช้สารประกอบซัลไฟต์ และการผลิตโปรตีนถั่วเขียวเชิงการค้าเพื่อเป็นอาหารมนุษย์ เนื่องจากปัจจุบันตลาดต่างประเทศมีความต้องการโปรตีนพืชสูงมาก และประเทศไทยใช้โปรตีนถั่วเขียวเข้มข้นจากกระบวนการผลิตสตาร์ชถั่วเขียวและวุ้นเส้นเป็นแหล่งอาหารโปรตีนสำหรับเลี้ยงสัตว์ ไม่ได้ใช้เป็นอาหารมนุษย์ ความสามารถในการออกแบบเมทริกซ์ของอาหารโปรตีนสูงชนิดมัลติเฟสที่ผ่านกระบวนการทำแห้งให้มีความคงตัวระหว่างการเก็บรักษาและควบคุมอัตราการย่อยสารอาหารเพื่อให้ปลดปล่อยสารออกฤทธิ์ตามเวลาที่ต้องการได้