ENZYMATIC HYDROLYSIS OF DEER PLASMA AND ITS EFFECT ON HYDROLYSATE ANTIOXIDANT ACTIVITY

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Abstract – Hydrolysis of proteins by proteases breaks them down into smaller peptides with reportedly higher bioactivity. In this study, a variety of protease extracts were used to hydrolyze deer plasma proteins into smaller fragments. Protein profiles of the deer plasma hydrolysates are shown on SDS-PAGE. DPPH scavenging activity of some hydrolysates showed a marked increase after hydrolysis. ORAC activity also increased while FRAP did not show any appreciable change. The use of proteases to hydrolyse deer plasma augmented the DPPH and ORAC antioxidant activity of the substrate.

Key Words – antioxidant activity, deer plasma, protease hydrolysis

I. INTRODUCTION

Blood is a significant by-product of the animal slaughterhouse industry. The recovery and extraction of bioactive compounds from slaughterhouse blood, after suitable collection, has been seen as an opportunity to add economic value and generate new applications for this by-product. Hydrolysis of blood proteins has been shown to generate peptides with higher antioxidant activity. The extent of hydrolysis of blood and the potency of bioactive peptides can be influenced by the choice of protease and the hydrolysis conditions including enzyme-to-substrate ratio, temperature, pH, and time of hydrolysis [1]. Peptide size and amino acid sequence also dictate the type of exerted bioactivity. Deer processing and venison production is a significant industry in New Zealand which provides a valuable potential resource (deer blood) for study. In this paper, the effect of hydrolysis using various proteases on the antioxidant activity of deer plasma is reported.

II. MATERIALS AND METHODS

(i) Materials

Fresh deer blood was obtained from a local meat processing facility – Alliance Group Ltd. Blood was collected with sodium citrate (3g/L) added to prevent coagulation and centrifuged at 3,000 rpm, 4°C for 15 minutes to separate the red blood cells from the plasma fraction. The plasma fraction was stored at -20 °C until use.

All chemicals used were of analytical reagent grade unless otherwise stated. Papain 25,000 MG (a papaya latex powder preparation), bromelain 110 (a pineapple fruit powder preparation), fungal protease 31,000 (F31K), fungal protease 60,000 (F60K), bacterial protease G (ProG), and fungal protease II (FPII), were kindly provided by Enzyme Solutions Pty. Ltd. (Australia).

Fresh New Zealand kiwifruit (*Actinidia deliciosa*) was obtained from a local market. Trypsin (1,800 BAEE units / mg solid) was from Sigma-Aldrich® while Flavourzyme (1000 LAPU/g) was from Novozyme, Denmark. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, fluorescein sodium salt, and 2,4,6-tripyridyl-s-triazine (TPTZ) were obtained from Sigma-Aldrich® (USA). 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Cayman Chemicals (USA).

(ii) Kiwifruit extract preparation

Kiwifruit pulp was homogenised using a blender. The juice was centrifuged at 14,000 rpm (IEC Micromax) for 10 minutes. The clarified supernatant was stored frozen at -20°C until use.

(iii) Deer Blood Plasma Hydrolysis

Based on preliminary experiments, hydrolysis was carried out over 24 hours with a second aliquot of protease added 8 hours after the start of hydrolysis. The hydrolysis conditions are given in Table 1. The pH of deer plasma was adjusted for the protease being used, by addition of 6N HCl. Protease-only controls were dissolved in 100 mM phosphate buffer solutions corresponding to the pH given in Table 1.

Table 1. Hydrolysis conditions

Protease	pH	Temperature	Concentration
Fungal 31,000	7.0	45°C	20 mg/ml[a]
Fungal 60,000	7.0	45°C	20 mg/ml[a]
Protease G	8.0	45°C	20 mg/ml[a]
Fungal Protease II	8.0	45°C	20 mg/ml[a]
Papain	8.0	45°C	4 mg/ml[a]
Bromelain	6.0	45°C	10 mg/ml[a]
Kiwifruit extract	6.0	45°C	1:2 (v/v)[b]
Trypsin	8.0	45°C	4 mg/ml[a]
Flavourzyme	8.0	45°C	1:2 (v/v)[b]

[a]Dry weight of protease added per volume of plasma
[b]Volume of protease solution added to volume of plasma

(iv) Protein profile of hydrolysates

Proteins were displayed by 1D-SDS–PAGE using BOLT™ gradient (4 – 12%) Bis–Tris gels (Invitrogen, Christchurch, NZ). A 2 µl aliquot of each plasma sample or plasma hydrolysate (diluted 5-fold) was added to 3.8 µl of SDS sample buffer, 1.5 µl of reducing agent and 8 µl of Milli-Q water. The samples were incubated at 90°C for 5 min prior to being loaded on a gel. Electrophoresis was performed in BOLT SDS running buffer (1x) at 160 V for 30 – 35 min at room temperature. Protein standards (Novex Sharp Pre-stained Protein Standard, Invitrogen, New Zealand) were included in the gel as a molecular marker. After electrophoresis, the gels were stained in SimplyBlue™ SafeStain (Invitrogen) and then de-stained with Milli-Q water.

(v) DPPH radical scavenging assay

DPPH radical scavenging activity was carried out as described by [2], with reaction times of 1 hour and 24 hours. As precipitates were formed in the samples when the hydrolysates were added to the DPPH-methanol solution, the samples were centrifuged prior to measurement of absorbance [3]. The DPPH free radical scavenging activity was calculated using the following formula:

$$\%\ radical\ scavenging = \left(\frac{(C - Cb) - (S - Sb)}{C - Cb} \right) \times 100$$

Where C is the absorbance of water plus DPPH in methanol,
Cb is the absorbance of water plus methanol,
S is the absorbance of the sample added to DPPH in methanol,
Sb is the absorbance of the sample plus methanol.

(vi) ORAC assay

The oxygen radical antioxidant capacity (ORAC) assay uses 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator and reports the antioxidant activity of a compound relative to a standard antioxidant (Trolox) [4]. Deer plasma hydrolysates and protease-only controls were diluted in 75 mM PBS (up to 300-fold depending on sample) prior to assay.

(vii) FRAP assay

The ferric reducing ability of plasma (FRAP) assay [5] was performed in a 96-well microplate. Test samples (20 µl) were mixed with 150 µl FRAP solution. FRAP solution was prepared by mixing acetate buffer, ferric chloride hexahydrate ($FeCl_3.6H_2O$) and TPTZ, at a ratio of 10:1:1 respectively. The mixture was mixed and incubated at 37°C. Absorbance was measured at 595 nm after 60 min. The change of absorbance was compared to that of a standard that was run concurrently. Standards of known Fe (II) concentrations ($FeSO_4.7H_2O$) were run in triplicate using several concentrations between 25 to 1000 µM (working concentration). A standard curve was then prepared by plotting the average FRAP value for each standard versus its concentration. The FRAP values for the samples were then determined using this standard curve [6].

III. RESULTS AND DISCUSSION

The hydrolysis of deer plasma proteins using a selection of proteases varied depending on the protease used as evident in Figure 1. The most abundant protein in plasma, albumin, was more successfully hydrolysed by F31K, FPII, papain, kiwifruit extract, trypsin and Flavourzyme into

smaller peptides compared to F60K, ProG and bromelain. The proteases alone did not contribute to the band profile on SDS-PAGE (data not shown).

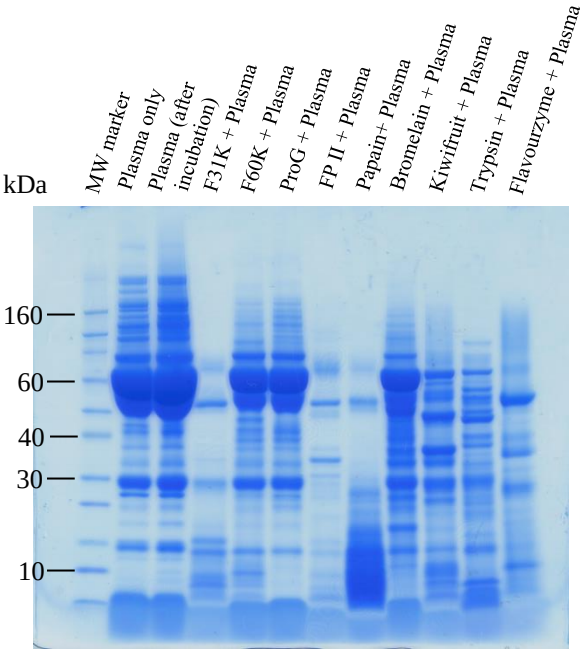

Figure 1. 1D-SDS-PAGE Protein profile of deer plasma hydrolysates

Antioxidant activity of the deer plasma protein hydrolysates after hydrolysis in terms of DPPH radical scavenging activity increased compared to the untreated plasma only controls (Figure 2A). After 24 hours of reaction time, significant DPPH radical scavenging activity was generated by the F31K, FPII, Kiwifruit and Flavourzyme treated plasma samples, which correlated with increased hydrolysis of proteins visualised on SDS-PAGE. However, the corresponding protease-only controls (F31K, FPII, Kiwifruit and Flavourzyme also demonstrated strong DPPH radical scavenging activity (Figure 2B). The apparent strong antioxidant activity of the proteases could be contributing and potentially masking the activity of the deer plasma hydrolysates.

The increase in DPPH radical scavenging activity of the deer plasma hydrolysates after hydrolysis compared to untreated deer plasma is more evident in samples treated with bromelain and trypsin as the protease-only controls demonstrated low DPPH radical scavenging activity. Bromelain + plasma had the highest activity (80.2 ± 1.1%) after 24 hours of reaction with DPPH, followed by trypsin + plasma (57.8 ± 5.4%), compared to the plasma only control (42.4 ± 2.6%). Interestingly, the plasma only control had a higher DPPH radical scavenging activity after incubation at 45°C compared to the frozen plasma sample (31.7 ± 2.6%) suggesting auto proteolysis of the deer plasma had occurred during incubation.

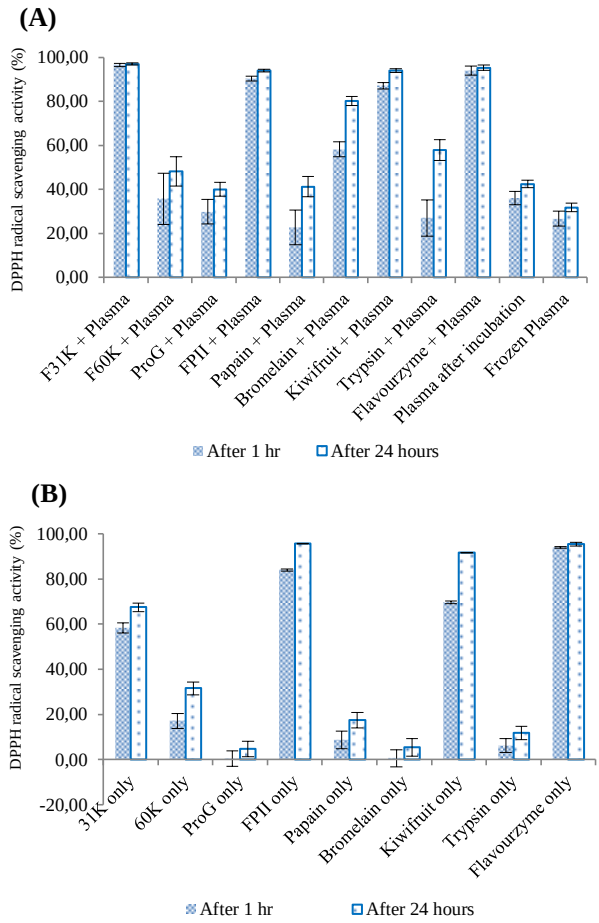

Figure 2. DPPH radical scavenging activity of deer plasma hydrolysates (A) and enzyme only controls (B)

While the profile of the bromelain + plasma hydrolysate on SDS-PAGE (Figure 1) did not appear to be as hydrolysed as the other hydrolysates, the DPPH activity was still high; suggesting that an intermediate sized peptide(s) could be responsible for the antioxidant activity.

ORAC activity of deer plasma (expressed as µM Trolox Equivalent (TE)) increased after hydrolysis with proteases compared to the untreated plasma

only controls. The highest ORAC activity was found for trypsin + plasma and Flavourzyme + plasma hydrolysates (Figure 3), followed by F31K + plasma, FPII + plasma and bromelain + plasma. Of the enzyme only controls, FPII and Flavourzyme had high ORAC activity.

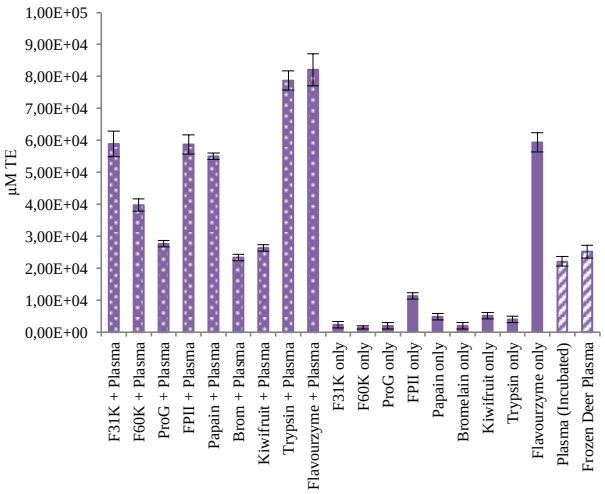

Figure 3. ORAC activity of deer plasma hydrolysates and enzyme only controls

The FRAP assay utilizes the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (Fe^{III} – TPTZ) complex and produce a coloured ferrous (Fe^{II} – TPTZ) form [5]. FRAP activity of the deer plasma was found not to be affected by hydrolysis as the FRAP value of the untreated deer plasma was similar to treated deer plasma hydrolysates (Figure 4).

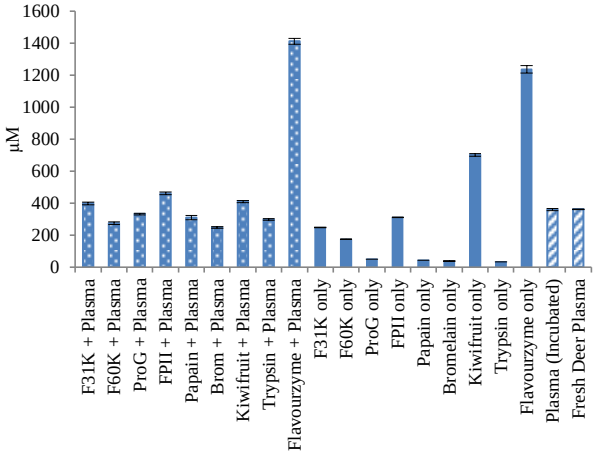

Figure 4. FRAP activity of deer plasma hydrolysates and enzyme only controls

IV. CONCLUSION

This study has indicated that the antioxidant activities (DPPH and ORAC) of deer plasma can be augmented by protein hydrolysis using proteases. Further studies on changing and optimizing hydrolysis conditions (e.g. different temperatures, pH, concentration, and length of time) to obtain optimal antioxidant activity and identifying the peptides responsible for antioxidant activity, are underway.

ACKNOWLEDGEMENTS

The authors thank Alliance Group Ltd. and Enzyme Solutions Pty. Ltd. for supplying deer blood and proteases, respectively.

REFERENCES

1. Bah, C.S.F., Bekhit, A.E.D., Carne, A. & McConnell, M. (2013). Slaughterhouse Blood: An Emerging Source of Bioactive Compounds. Comprehensive Reviews in Food Science and Food Safety 12: 314 - 331.
2. Brand-Williams, W., Cuvelier, M.E. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology 28: 25-30.
3. Janaszewska, A. & Bartosz, G. (2002). Assay of total antioxidant capacity: comparison of four methods as applied to human blood plasma. Scandinavian Journal of Clinical & Laboratory Investigation 62: 231-236.
4. Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A. & Prior, R.L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. Journal of Agricultural and Food Chemistry 50: 4437-4444.
5. Benzie, I.F. & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 239: 70-76.
6. Griffin, S.P. & Bhagooli, R. (2004). Measuring antioxidant potential in corals using the FRAP assay. Journal of Experimental Marine Biology and Ecology 302: 201-211.