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Analytical Methods

Development of 4-hydroxyproline analysis kit and its application to collagen quantification

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ABSTRACT

4-Hydroxyproline (4-Hyp) is a specific amino acid of collagen and widely used as a factor to estimate the collagen content in biological specimens. The authors have developed an analysis kit with the ability to detect collagen on microwells. The method includes chromophore formation without solvent transfers, that allows the analysis of multiple specimens with excellent sensitivity, high specificity at low cost with shorter analysis time. The calibration curve of 4-HYP kit exhibiting a high positive relationship ($R^2 = 0.999$) while showing a very low detection limit of (1.0 μg/ml). Specificity of 4-HYP kit was decreased with increasing hydrolysed non-collagenous biomolecules (HNCB), however this was negligible since only a few collagen specimens have a high amount of HNCB. The 4-HYP kit was successfully applied to commercial collagen quantification, measuring the collagen content of connective tissue and collagen synthesis of fibroblast with high satisfactory results; therefore, this is a more suitable alternative to previous analysis methods.

1. Introduction

Collagen is major component of mammalian connective tissue, accounting for approximately 30% of all protein in the human body (Lin & Liu, 2006). It can be fabricated into various forms such as a gel, sponge, fibre, and film to serve as a scaffold for tissue engineering with unique biocompatibility and biodegradable properties (Angele et al., 2004; Lin & Liu, 2007; Pachence, 1996). There are at least 26 types of collagen that were identified (Ricard-Blum & Ruggiero, 2005), and these collagens are distributed differently in most organs and tissues. The primary structure of polypeptide chains of collagen exhibit a repeating sequence of Gly–Xaa–Yaa structural motif in the entire peptide chain, where Xaa and Yaa are usually substituted by a 4-Hyp and proline. Due to the unique repeating sequence of collagen, the typical amino acid composition of collagen exhibits 33% of glycine, 10% of proline and 14% of 4-Hyp.

Considering the role of collagen and its significance in biochemistry, the demand for a technique of collagen quantification has become important. Methods for the determination of collagen content in biological specimens can be classified into several groups. Microassay methods have been used to quantitate *in vitro* collagen synthesis by the amount of [^{3}H] proline incorporation (Diegelmann, Bryson, Flood, & Graham, 1990; Kirchhofer, Reinhardt, & Zbinden, 1986). However this analysis method was limited because of the tedious procedures and risk of radiation poisoning. One technique reported, was based on an immunochemical approach, this assay is a competitive EIA (enzyme immunoassay) or ELISA (enzyme-linked immunosorbent assay) in which polyclonal antibody to human type I atelo-collagen is used. This approach provided a reliable and sensitive methodology. However, to obtain results, monospecific antibodies requires the various collagens to be purified to homogeneity and this is difficult to achieve (Quasnichka et al., 2005). ELISA kit was limited on commercial application due tedious procedures, expensive price, and species-specific problems. Colgrave, Allingham, and Jones (2008) proposed a novel method utilising a highly selective and sensitive method of multiple reactions monitoring (MRM) by mass spectrometry. The capillary electrophoresis was also used to determine 4-Hyp content of bovine skeletal perimysial collagen preparations and whole muscle samples (Chu, Evans, & Zeece, 1997; Chu & Zeece, 2000). However, these analysis methods were limited due to the expensive instruments used, tedious derivation procedures and low detectable range.

Walsh, Thornton, Penny, and Breit (1992) proposed a collagen quantification method based on the mechanism of formation of specific dye-collagen precipitate using Sirius Red. Sirius Red is an anionic dye with sulphonic acid side chain groups. These groups react with the side chain groups of the basic amino acids present

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in collagen. The specific affinity of the dye for collagen, under assay conditions is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagens that have triple helix organisation intact and is determined by their absorbance at 540 nm. However the Sirius Red may bind with serum albumin and overestimate the amount of collagen present (Marotta & Martino, 1985; Walsh et al., 1992).

Nevertheless, a convenient, accurate determination method of collagen content is required. In order to simplify and optimise the analysis method of 4-HYP, authors proposed a novel 4-Hyp analysis kit (4-HYP kit) for collagen quantification. The sensitivity, specificity, coating recovery of the analysis kit and determination of biological specimen were conducted. Moreover, the authors used a commercial collagen assay kit as the gold standard to compare the sensitivity and specificity of the 4-HYP kit. The authors hope to provide better alternative to conventional analysis methods to be applied on biotechnology and food analysis.

2. Materials and methods

2.1. Preparation of reagents

A solution containing 1 mg/ml of 4-Hyp was dissolved in ultrapure water as 4-Hyp stock solution. The 4-Hyp stock solution was diluted to 0, 20, 30, 50, 60, 70, 80, and 100 μg/ml (4-Hyp/ultrapure water) by serial dilution and used as 4-Hyp standard solution. Acetate–citrate buffer (pH 6.5) was prepared by dissolving 12 g of sodium acetate trihydrate. 4.6 g of citric acid, 1.2 ml acetic acid, and 3.4 g of sodium hydroxide in ultrapure water; pH value was adjusted to 6.5 and brought to 100 ml. Chloromine T reagent was prepared by dissolving 1.27 g of chloromine T in 20 ml 50% *n*-propanol and brought to 100 ml with acetate–citrate buffer; since this reagent is not stable, it should be prepared fresh before each assay and stored in a light-tight container. Ehrlich's reagent was prepared by 15 g of 4-(dimetylamino)-benzaldehyde dissolving in *n*-propanol/perchloric acid solution (2:1, v/v) and brought to 100 ml; since this reagent is not stable, it should be prepared fresh before each assay and stored in a light-tight container.

2.2. Development of standard operational procedures

The standard operational procedures were optimised according to the methods described by Reddy and Enwemeka (1996) and Ignat'eva et al. (2007). The standard operational procedures are stated as follows. The specimens were hydrolysed at 120 °C for 40 min by autoclave (B25HM, Tomin Ltd., Taiwan). Twenty microlitres of standard 4-Hyp solution (0–100 μg/ml) and test samples were added to a 48 wells Multidish (150687, Nunc, Denmark) and mixed with 30 μl of sodium hydroxide solution (to 2 N final concentration). This was then mixed with 450 μl of buffered chloromines T reagent and oxidation proceeded for 25 min at room temperature. The chromophore was developed with the addition 500 μl of Ehrlich's reagent and was incubated at 65 °C for 40 min by dry bath incubator (MD-02N-110, Major science, Taiwan). As the absorbance of chromophore exhibits instability at high temperatures, a "gradual cooling cycle" method was adapted for 4-Hyp analysis. The incubated multiplates were gradually cooled down according a time cycle of 10–15–20 min (room temperature – 4 °C – room temperature). Finally, the absorbance of specimens was detected at 550 nm using ELISA reader (MicroQuant; Bio-Tek. Instruments, USA).

2.3. Detection of limit and accuracy

The 4-Hyp standard solution was serial diluted to 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 17.5, 20.0, 30.0, 50.0, 60.0, 70.0, 80.0, and 100.0 μg/ml (4-Hyp/ultrapure water) and regarded as a test specimen. The specimen was analysed according to standard operational procedures. The accuracy of 4-HYP kit in various concentrations was calculated by the following formulation:

$$\text{Accuracy } (\%) = \frac{\text{Detected amount of 4-HYP concentration}}{\text{Loaded amount of 4-HYP concentration}} \times 100.$$

When the measured results ± standard derivation ranged within 100 ± 5%, these were regarded as passing the accuracy test and, used in the detection of 4-Hyp at specific concentrations.

2.4. Specificity

To detect the inference of hydrolysed non-collagenous biomolecules (HNCB) on the measurement of 4-HYP kit, 450 μl of biomolecule solution (bovine serum albumin, chondroitin-6-sulphate and hyaluronic acid) was added to 450 μl of 3.3 N NaOH solution and hydrolysed at 121 °C for 40 min. The HNCB solutions were co-added with 4-Hyp standard solution in different relative ratios (biomolecule concentration/4-Hyp concentration = 0.1%, 1.0%, 10.0%, and 100.0%) and loaded into the microwell of multiplate, mixture and analysed by standard operation procedures of 4-HYP kit. The suppression of the HNBP on the reaction of 4-HYP kit was calculated by the following formulation:

$$\text{Specificity } (\%) = \frac{\text{Absorbance of 4-HYP standard solution with HNGB}}{\text{Absorbance of 4-HYP standard solution without HNGB}} \times 100.$$

When the measured results ± standard derivation ranged within 100 ± 5%, this indicated that the 4-Hyp was correctly measured by 4-HYP kit at a specific concentration.

2.5. Coating recovery

To reduce the tedious procedures of 4-Hyp standard solution dilution and random errors, the authors wanted to develop a user-friendly interface in which the user applies a 4-HYP kit without dilution of standard solutions. The microplate was pre-coated with an equal mass of 4-Hyp standard solution. In order to promote the diffusion of 4-Hyp and reaction between sample and reagents, several coating agents: 0.1% sucrose, 0.1% sodium chloride, 1% sodium acetate, 1% SDS (sodium dodecyl sulphate), 0.1% Triton X-100, 1% Triton X-100, 0.1% glycine, and 0.1% serine were dissolved with 95% ethanol. Two-hundred microlitres of coating agent/95% ethanol was added on the microwells of multiplate, the ethanol was evaporated in a laminar flow at room temperature. The mixture of 4-Hyp and 95% ethanol will form a porous 4-Hyp film on wells of the microwells and rapidly dissolve without specific interactions. To evaluate the efficiency of coating agents, the coating recovery was calculated by the following formulation:

$$\text{Coating recovery } (\%) = \frac{\text{Absorbance of microwell pre-coated with coating agent}}{\text{Absorbance of 4-Hyp added alone}} \times 100.$$

The coating recovery was classified as no effect (98–100%), slight effect (90–98%), medium effect (75–90%) and large effect (50–75%), respectively.

2.6. Application on animal connective tissue

The application of the 4-HYP kit was examined using connective tissue from genetically diabetic mice (NOD/Ltj). The genetically diabetic mice were sacrificed by CO_2 euthanasia, the brain, heart, kidney, liver, lung, muscle (*biceps femoris*) and testis were

dissected by Iris scissor. The organs were immediately immersed in linger solution, in an ice bath, the linger solution was removed by using a suction unit (TM-330, Tomin medical equipment CO., Taiwan) and replaced by distilled water three times. The organs were frozen at −86 °C, freeze dried and pulverised by using a mortar. The organ powder was re-suspended in 2 N NaOH (1%, w/v) and hydrolysed at 121 °C for 40 min, 20 μl of tissue hydrolytes were added to microwells and analysed according to standard operation procedures of 4-HYP kit as previously described. The collagen content was calculated assuming 14% of collagen is 4-Hyp.

2.7. Measurement of collagen synthesis by human fibroblast

Human fibroblasts (HS68) were purchased from Bioresource Collection and Research Centre (BCRC, Taiwan), cultures were established in 25 cm^2 culture flasks in DMEM supplemented with 10% FBS, 2 mM glutamine, and 50 μg/ml gentamycin, as described previously (Freshney, 2000). Monolayer cultures were maintained at 37 °C in 5% CO_2/air. Fibroblasts between the third and sixth subpassages were used for the experiments. Until subconfluence, the fibroblast were harvested by trypsinisation, viable cells were counted by using a dye excluding method with seeding on a round tissue plate that had a density of $0.6 \times 10^4/cm^2$, which was maintained at 37 °C in 5% CO_2/air for 3, 5, 7, and 9 days, respectively. The cell layer on tissue culture flask was trypsinised by 5 ml of 0.25% trypsin/EDTA solution at 37 °C for 20 min and collected as cell lysate solution. The specimens were hydrolysed by 2 N NaOH at 121 °C for 40 min and measured by 4-HYP kit by mentioned standard operational procedures.

2.8. Comparison of 4-HYP kit and commercialised collagen assay kit

Sircol™ collagen assay kit (Biocolor Ltd., United Kingdom) was purchased from a local distributor; the porcine pepsin-soluble collagen (Nippon Ham, Japan) was kindly provided by Professor Kuroyangi and used as a standard. Two-hundred microlitres of specimen were added into microcentrifuge tubes and 1.0 ml Sircol dye reagent was added to all tubes. Tubes were capped and the contents were mixed by using a mechanical mixer at room temperature for 30 min. Tubes were transferred to a microcentrifuge and centrifuged at 11,000 *g* for 10 min to obtain collagen-dye pellet at the bottom of the tubes. To remove the unbound dye solution, the supernatants were drained off and discarded. Absorbent paper tissue was used to remove any dye solution from the top end of the tube wall. To the collagen-dye pellet, 1.0 ml of the alkali reagent was added, then the tubes were capped and contents mixed by a mechanical mixer at room temperature for 10 min and the collagen bound dye was dissolved into alkali reagent. Finally, the dye solutions were added into 98 wells multiplate and determined at 540 nm by ELISA reader (MicroQuant; Bio-Tek. Instruments, USA).

To verify the specificity of Sircol™ collagen assay kit, several biomolecules (bovine serum albumin, chondroitin-6-sulphate and hyaluronic acid) were co-added with the collagen solution at different relative ratios (biomolecule concentration/collagen concentration = 1.0%, 10.0%, and 100%) and the detection method was carried out by standard operational procedures of the user manual.

3. Results and discussion

3.1. Development of standard operational procedures

The entire procedure of 4-HYP kit comprises of five common steps: (a) hydrolysis by NaOH solution, (b) oxidation by chloromine T reagent, (c) development of chromophore, (d) cool down, and (e) measuring absorbance at 550 nm. The calibration curve was obtained by using a standard 4-Hyp solution. The results demonstrated that the absorbance is linearly related ($R^2 = 0.999$) to the amount of 4-Hyp over the range of 0–100 μg/ml, the linear regression equation of A550 = −30.1 + (390 × 4-Hyp concentration) were obtained. In previous studies, the detected range for 4-Hyp were 0–20 μg/ml (Reddy & Enwemeka, 1996) and 0–1.5 μg/ml (Ignat'eva et al., 2007), respectively. However, the detectable range of 4-HYP kit was greater than in previous studies (Ignat'eva et al., 2007; Reddy & Enwemeka, 1996) and exhibited a higher regression coefficient ($R^2 = 0.999$). Furthermore, using two 48 wells multiplate −8 standard specimens per plate, the 4-HYP kit can measure 80 specimens simultaneously. 4-HYP kit reduced analysis time (within 4 h) more than the previous 4-Hyp analysis method which may provide a useful tool for food and biomedical research areas.

3.2. Type I collagen determination

Soluble porcine type I collagen was used for verifying the correlation between 4-Hyp concentration (μg/ml) and collagen content (%) ($R = 0.988$). The standard curve gave good reproducibility and strict relation between 4-Hyp concentration (μg/ml) and collagen content (%). Standard deviations in quadruplicates did not exceed 5%. The relationship between 4-Hyp solution and commercial type I collagen was established. Results demonstrated that the collagen concentration is linearly related to the amount of 4-Hyp, the linear regression equation: Collagen (%) = −655 + (20.7 × 4-Hyp concentration) was obtained.

3.3. Detection of limit and accuracy

In analytical chemistry, the limit of detection (LOD) is the lowest quantity of a substance that can be distinguished from the absence of that substance within a stated confidence limit (generally 5%). The 4-HYP kit exhibited sensitive and reproducible results, the detected 4-Hyp concentration were consistent with loaded 4-Hyp concentration (Fig. 1). The accuracy of 4-HYP kit exhibited satisfactory results and ranged within a confidence limit (±5%) in various 4-Hyp concentrations (Fig. 1). Moreover, 4-HYP kit can detect 4-Hyp at an extremely low concentration (1 μg/ml) and provide a precise result (accuracy = 100%). In addition, the 4-HYP kit can provide a reliable analytical performance without expensive instruments and tedious analysis procedures.

3.4. Specificity

Connective tissues contain a large amount of glycosaminoglycans such as C6S and HA which are undegradable under severe hydrolysis (Ignat'eva et al., 2007). Moreover, the serum albumin is a major compound in animal serum, which may interfere in the reaction between 4-Hyp and Ehrlich's reagent. The authors performed an experiment to study the effect of above biomolecules on the analysis result of the 4-HYP kit, the hydrolysed biopolymers were mixed with 4-Hyp standard solution in different relative ratio (biomolecule concentration/4-Hyp concentration = 0.1%, 1.0%, 10.0%, and 100%) and analysed by standard operational procedures with a 4-HYP kit. The interference of hydrolysed non-collagenous biomolecules (HNCB) on specificity of 4-HYP kit was dose dependent (Fig. 2). Generally, the specificity was increasing in the order: HA > BSA > C6S. The lower specificity that appeared, was C6S, equal to 4-Hyp (C6S/4-Hyp = 100%) (Fig. 2). This result corresponded with a previous study (Ignat'eva et al., 2007). Since type II collagen has a major extracellular matrix of cartilage, accompanied with a large amount of C6S, it is undegradable, even under severe conditions (121 °C for 40 min). Due to the chemical structure of C6S lacking pyrrolidine and not reacting with Ehrlich's reagent, the authors assumed the phenomenon as related to the reactive group

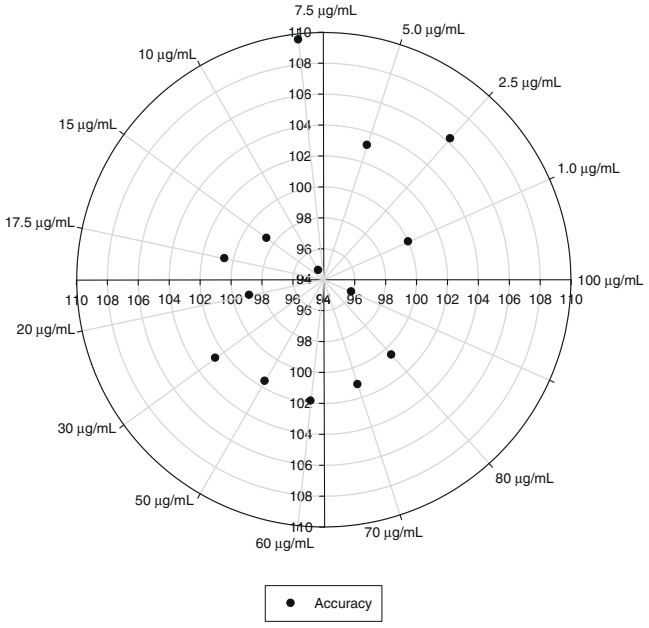

Fig. 1. Accuracy of 4-HYP kit over a range of 4-Hyp concentrations. When the measured results ± standard derivation ranged within 100 ± 5%, these were regarded as passing accuracy test and were allowed to detect 4-Hyp at specific concentrations.

between 4-Hyp and reagents that were stereometrically occupied by C6S.

3.5. Coating recovery

The internal standard curve is a necessary procedure for quantitative chemistry; however, the preparation and dilution of standard solutions may be prone to errors. Based on the above reasons, the authors would like to develop a "user friendly kit" in which the specific concentration of 4-Hyp is immediately liberated from the surface of microwells and reacted with reagents. Several coating agents were used to promote the releasing efficiency of 4-Hyp without non-specific reactions. Sodium chloride and sucrose provides a large amount of ion and hydrophilic groups. Sodium acetate is a major component of acetate–citrate buffer and exhibits high solubility within ethanol. SDS and Triton X-100 are typical surfactant used to giving the 4-Hyp higher amphiphilic properties and promoting solubility of 4-Hyp.

The results were shown in Table 1. The multiplate directly coated with 4-Hyp could not provide precise results (83.5 ± 2.16%). This result also implies that the multiplate coated with 4-Hyp alone is not a suitable method for 4-Hyp, as it would induce an underestimation of 4-Hyp concentration. The 4-Hyp seems precipitated with 0.1% NaCl (80.5 ± 18.9%) and 0.1% sucrose (76.9 ± 34.6%). Unexpectedly, the multiplate coated with 1.0% SDS (61.2 ± 23.4%), 0.1% Triton X-100 (68.1 ± 19.1%), and 1% Triton X-100 (63.4 ± 2.33%) could not provide a sufficient recovery. The non-polar amino acid (serine) used to coat a surface of multiplate exhibited an inconsistent result (94.1 ± 4.42%). Glycine is the most abundant amino acid of collagen and may be an ideal coating agent for the 4-HYP kit; however, the multiplate coated with 0.1% glycine exhibited a doubtful result (88.6 ± 2.07%).

Interestingly, the multiplate which was coated with 1% sodium acetate exhibited the highest recovery (98.2 ± 2.21%). However, the multiplate coated with low concentration sodium acetate (0.01%) did not provide a satisfactory recovery (90.8 ± 5.85%). The sodium acetate can provide the conjugate base (acetate) and conjugate

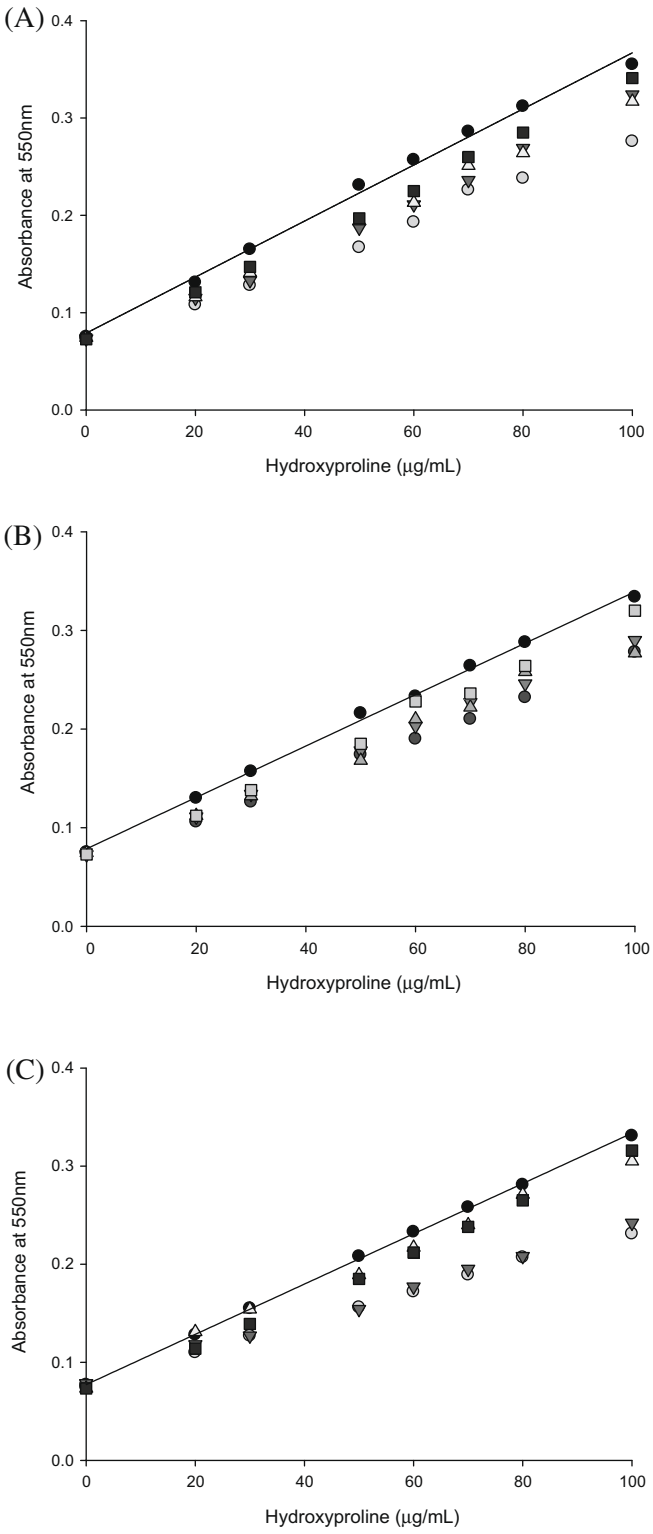

Fig. 2. Specificity of 4-HYP kit analysed over a range of concentrations (● = 0%, ▲ = 0.1%, □ = 1.0%, ■ = 10.0%, ▼ = 100%) of hydrolysed non-collagenous biomolecule (A) BSA, (B) HA, and (C) C6S.

acid (acetic acid) and act as a buffer to keep a relatively constant pH and is widely using in a variety of fields (Lehninger, Nelson, & Cox, 2008). The sodium acetate is easily soluble in ethanol solution and saturated until 3 M. The high ethanol solubility of sodium acetate may be promoted by the 4-Hyp dispersion and solubility during analysis. Furthermore, the sodium acetate is a major

Table 1
Recovery of plate microwells coated with different coating agents.

Coating reagent	Recovery (%)	SD (%)	Effect
4-HYP alone	83.5	2.16	Medium effect
0.1% NaCl	80.5	18.9	Medium effect
0.1% sucrose	76.9	34.6	Medium effect
0.01% sodium acetate	90.8	5.85	Slight effect
1% sodium acetate	98.3	2.21	No effect
1% SDS	61.2	23.4	Large effect
0.1% Triton X-100	68.1	19.1	Large effect
1% Triton X-100	63.4	2.33	Large effect
0.1% glycine	88.6	2.07	Medium effect
0.1% serine	94.1	4.42	Slight effect

component of acetate–citrate buffer and would be more compatible with the 4-HYP kit. The authors thought that the 1% sodium acetate would be an optimum coating agent for 4-HYP kit and provide more convenient and effective approach for routine analysis.

3.6. Application on animal connective tissue

The applications for the 4-HYP kit for tissue sample from the brain, heart, kidney, ear, liver, lung, muscle, and testis from NOD/Ltj mice. Table 2 represents the results and the collagen content of tissue were increasing in order of ear > lung > testis > heart > liver > muscle > kidney > brain. In the present study, 4-Hyp values determined in these biological specimens were found to be in close agreement with conventional methods and other published reports (Edwards & O'Brien, 1980; Reddy & Enwemeka, 1996).

The collagen content is an important indicator in many metabolic diseases. Excessive production of collagen has been documented in proliferative disorders such as liver cirrhosis, lung fibrosis and tumour growth (Williams et al., 2001). Loss of tissue collagen has been observed in certain disorders of connective tissue including rheumatoid arthritis and wounded/ulcer damaged tissue (Reddy & Enwemeka, 1996). In food quality aspects, the insoluble hydroxyproline of muscle tissue not only adversely influenced textural properties contributing to tenderness, but also adversely influenced flavour desirability (Jeremiah, Dugan, Aalhus, & Gibson, 2003). The 4-HYP kit will be a useful tool for routine measurement of collagen content in connective tissue and can be applied in clinical diagnosis and meat quality control.

3.7. Measurement of collagen synthesis by human fibroblast

Measurement of collagen synthesis in fibroblast cultures is a common approach to examine specific aspects of cellular metabolic changes of normal and altered connective tissue caused by hereditary and acquired disease, hormones, drugs and other chemicals or environmental factors (Diegelmann et al., 1990; Tredget, Falk, Scott, Hogg, & Burke, 1990). The detected value of cultured plate which is treated by trypsinisation for 20 min consistent than the detected value of culture medium (date not shown). Under our experimental conditions collagen synthesis of fibroblast markedly increased during fibroblast proliferation (Fig. 3) and consequentially secreted into culture medium or accumulated in intercellular space. The results demonstrated that the population of fibroblast is linearly related ($R = 0.969$) to the concentration of 4-Hyp (Fig. 3), the linear regression equation of fibroblast number = $-133076.391 + (7915.503 \times$ 4-Hyp concentration). This result is consistent with previous studies (Diegelmann et al., 1990; Kirchhofer et al., 1986; Tullberg-Reinert & Jundt, 1999).

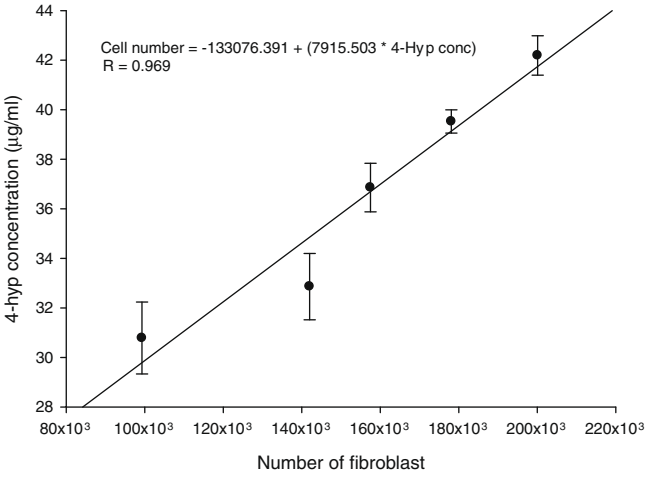

Fig. 3. Relationship between the number of fibroblasts and the 4-Hyp concentration.

Recently, animal cell culture became an important approach to evaluation of functions of food components (Bast & Haenen, 2002; Nayaka, Sathisha, Manohar, Chandrashekar, & Dharmesh, 2009; Shigemura et al., 2009). Moreover, the collagen synthesis of fibroblast is considered as an index of cellular ageing (Ravelojaona, Robert, & Robert, 2008). The collagen synthesis of connective tissue derived cell cultivation is also regarded as an indicator for cell proliferation (Qiao, Bell, Juliao, Li, & May, 2009; Shahdadfar et al., 2008). We demonstrated that fibroblasts incubated in culture medium can be synthesised into detectable amounts of collagenous proteins and detected by 4-HYP kit. The present study also supports the need for an economical assay for collagen synthesis where multiple measures can be determined conveniently.

Table 2
Measured 4-hydroxyproline and calculated collagen content (dry basis) of various organs from genetically diabetic mice.

Organ	Measured 4-Hyp content (µg/ml)[b] Mean ± SD	Calculated collagen content (%) Mean ± SD	Previous data of Reddy and Enwemeka (1996)[c] Mean	Previous data of Edwards and O'Brien (1980)[d] Mean
Liver	6.63 ± 1.31	1.18 ± 0.23	0.15	0.69
Heart	8.99 ± 1.82	1.61 ± 0.35	0.18	–[a]
Kidney	5.67 ± 0.76	1.04 ± 0.14	0.27	1.90
Ear	45.5 ± 3.27	8.24 ± 0.59	–	–
Brian	4.03 ± 1.00	0.74 ± 0.18	–	–
Muscle	6.50 ± 2.76	1.40 ± 0.56	–	–
Testis	12.6 ± 1.69	2.32 ± 0.36	0.26	–
Lung	22.6 ± 0.89	3.99 ± 0.09	0.30	–

[a] Data not shown.
[b] NOD/Ltj mice (%, by dry basis).
[c] Rabbit (%, by wet basis).
[d] Rat (%, by dry basis).

3.8. Comparison of 4-HYP kit and commercial collagen assay kit

The Sircol™ assay is a well-establish kit for the analysis of soluble collagens from mammalian tissues and collagens released into a culture medium by mammalian cells during *in vitro* culture. A major advantage of Sircol™ Assay is the combination of identification of collagen and collagen-producing cells *in situ* with subsequent spectrophotometric quantification of the dissolved stain. However, the influence of biomolecules such as BSA, C6S and HA on the specificity of Sircol™ collagen assay kit is still not clear.

The authors have determined the influence of BSA, C6S, and HA on the specificity of Sircol™ collagen assay kit. The results are illustrated in Fig. 4 and reveal the inference of non-collagenous biomolecule was increasing in order: BSA > HA ≧C6S. The highest absorbance (151% of collagen alone) which appeared was the 60 μg/ml collagen solution (BSA/collagen solution = 100%) (Fig. 4). The BSA causing significant effects on assay may be due to BSA being precipitated with Sirius Red and increasing absorbance at 540 nm. The presence of BSA would induce systematic errors and overestimate the value of collagen concentration. BSA is a major component of foetal bovine serum (FBS) and plays a critical role on cell differentiation and expansion (Freshney, 2000). However, the instruction of Sircol™ collagen assay kit mentioned following comments "culture medium with foetal bovine serum supplement to 5% does not interfere with the collagen assay. When higher serum supplements have been used, the increasing bulk of serum proteins, relative to the amount of collagen present, can cause problems". However, reducing the serum supplement to 5% may alter the cell attachment and retard cell growth rate (Freshney, 2000). To selectively remove the BSA from the test sample by affinity chromatography is possible but it also prolongs analysis time. Moreover, the presence of C6S and HA also influence the specificity of Sircol™ collagen assay kit, the specificity of C6S and HA treatment were inconsistent and varied with different concentrations. The analysis procedures of Sircol™ collagen assay kit are comprised of six common steps: (a) adding sircol dye, (b) mixing (or reaction), (c) centrifuging, (d) draining, (e) release of bound dye, and (f) measurement. The inconsistent results should be closely related to errors during removal of unbound dye. The major principle of Sircol™ collagen assay kit is based on the "dye-affinity" and "recovery of bound dye". However, the Sirius Red is an anionic dye and not only binds to collagen but also bound to the surface of test tube. Even Sircol dye reagent contains a surfactant to aid draining from a test tube; the unremovable dye solution still remained on the test tube and influences the determination value of Sircol™ collagen assay kit. The biological specimen or culture medium inevitably contains various non-collagenous biomolecules and interferes with the analysis result of Sircol™ collagen assay kit, and still remains to be overcome either mathematically or by spectrometric correction.

3.9. Description of 4-HYP kit and its advantages

A convenient, user friendly and sensitive analysis kit for collagen quantification was described. The 4-HYP kit based on the method of Edwards and O'Brien (1980) and Reddy and Enwemeka (1996). The modifications include the use of a multiplate instead of a glass tube, 4-Hyp standard directly coated on multiplate, reduced number of reagents, and ELISA reader used instead of spectrometer.

In summary, the 4-HYP kit for collagen quantification offer a number of advantages over other methods, which are based either on Sirius Red-based colorimetric microassay (Sircol™ collagen assay kit), HPLC method, traditional spectrometric method, immunological method or H^3 labelling assay: (1) the requirement of sample volume is considerably less than traditional methodology (Ignat'eva et al., 2007; Komsa-Penkova, Spirova, & Bechev, 1996; Walsh et al., 1992; Woessner, 1961); (2) the 4-HYP kit can simultaneously measure 80 specimens within 4 h, which substantially reduces the cost and analysis time of experiments; (3) can be *in situ* and measure specimens without standard dilution and tube transfers; therefore significantly reducing the random error and systematic errors during analysis; (4) more sensitive than previous methods and can detect 4-Hyp in low concentrations (1 μg 4-Hyp/ml); (5) 4-HYP kit can be used for the analysis of collagen concentration

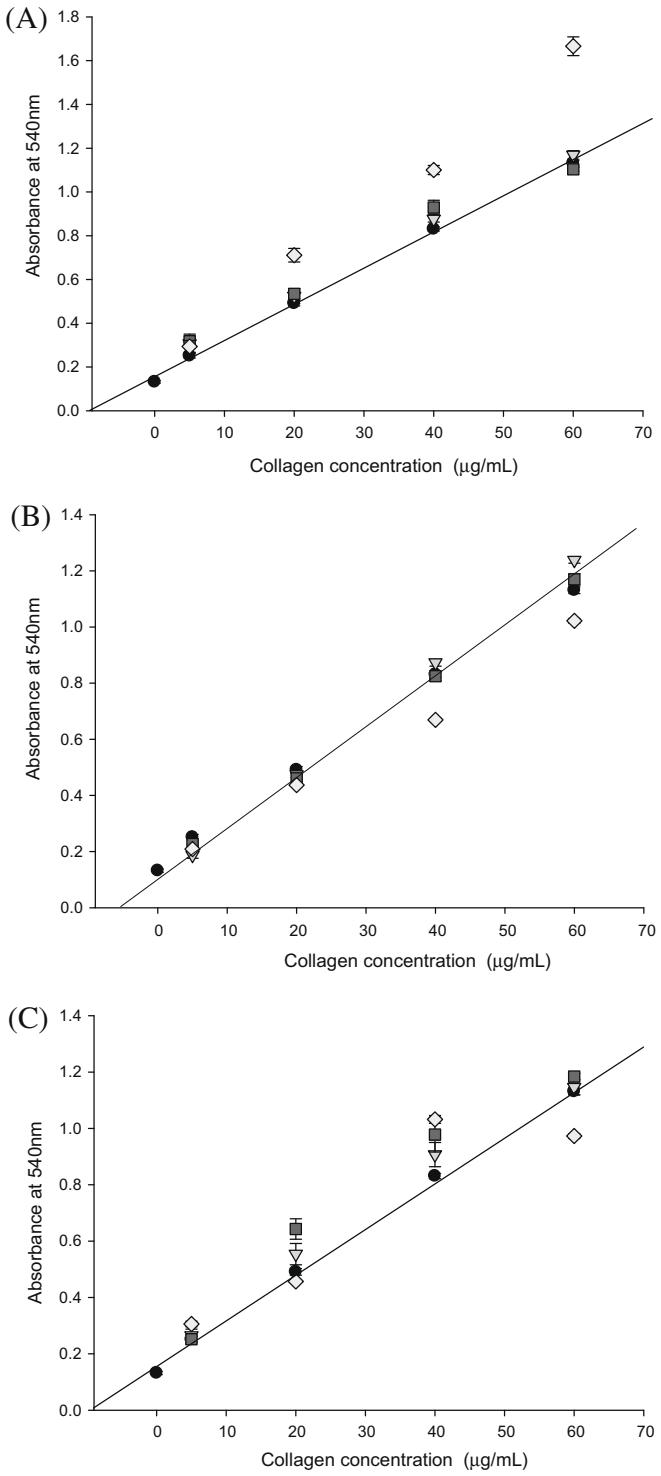

Fig. 4. Specificity of Sircol™ collagen assay kit which that analysed with various concentration (● = 0%, ▽ = 1.0%, ■ = 10.0%, ◇ = 100%) of noncollagenous biomolecule (A) BSA, (B) HA, and (C) C6S.

for any biological fluids, such as urine, plasma, tissue homogenates, or cell culture medium with high specificity results; and (6) it avoids the use of radio isotope tracer.

4. Conclusion

The limit of detection of 4-HYP kit was 1.0 μg/ml, A550 of specimen was increased as 4-Hyp content increased and exhibited a high positive relationship (R^2 = 0.999). The 4-HYP kit was successfully used with high sensitivity, high specificity and eliminated the time-consuming steps involved in a 4-HYP analysis. The multiplate wells pre-coated with 1% sodium acetate exhibited the highest recovery value during analysis, these results inspired the authors to develop a "user friendly kit" without the dilution of 4-Hyp standard solution. Specificity of analysis kit was decreased with HNCB increasing, but, the influence of HNCB was negligible due to only a few of biological samples containing such a high amount of HNCB and concomitant with collagen.

In the present study, a 4-HYP kit was successfully applied to commercial collagen quantification, measuring collagen content of connective tissue from diabetic mice and collagen synthesis of fibroblast. The 4-HYP kit, with the ability to detect collagen in microwells without transfers of material, allows the analysis of multiple specimens with excellent sensitivity and specificity at low cost and shorter analysis time. This kit is also useful for monitoring column fractions, cosmetic products and applicable to a wide range of studies in the field of collagen biochemistry.

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References

Angele, P., Abke, J., Kujat, R., Faltermeier, H., Schumann, D., Nerlich, M., et al. (2004). Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices. *Biomaterials, 25*(14), 2831–2841.

Bast, A., & Haenen, R. M. G. M. (2002). The toxicity of antioxidants and their metabolites. *Environmental Toxicology and Pharmacology, 11*(3–4), 251–258.

Chu, Q., Evans, B. T., & Zeece, M. G. (1997). Quantitative separation of 4-hydroxyproline from skeletal muscle collagen by micellar electrokinetic capillary electrophoresis. *Journal of Chromatography B – Biomedical Applications, 692*(2), 293–301.

Chu, Q., & Zeece, M. (2000). Capillary electrophoretic determination of 4-hydroxyproline. *Methods Molecular Biology, 159*, 227–235.

Colgrave, M. L., Allingham, P. G., & Jones, A. (2008). Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. *Journal of Chromatography A, 1212*(1–2), 150–153.

Diegelmann, R. F., Bryson, G. R., Flood, L. C., & Graham, M. F. (1990). A microassay to quantitate collagen synthesis by cells in culture. *Analytical Biochemistry, 186*(2), 296–300.

Edwards, C. A., & O'Brien, W. D. Jr., (1980). Modified assay for determination of hydroxyproline in a tissue hydrolyzate. *Clinica Chimica Acta, 104*(2), 161–167.

Freshney, R. I. (2000). *Culture of animal cells: A manual of basic technique*. Wiley-Liss.

Ignat'eva, N., Danilov, N., Averkiev, S., Obrezkova, M., Lunin, V., & Sobol', E. (2007). Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *Journal of Analytical Chemistry, 62*(1), 51–57.

Jeremiah, L. E., Dugan, M. E. R., Aalhus, J. L., & Gibson, L. L. (2003). Assessment of the relationship between chemical components and palatability of major beef muscles and muscle groups. *Meat Science, 65*(3), 1013–1019.

Kirchhofer, D., Reinhardt, C. A., & Zbinden, G. (1986). Collagen synthesis in growing human skin fibroblasts. *Experimental Cell Biology, 54*(4), 177–182.

Komsa-Penkova, R., Spirova, R., & Bechev, B. (1996). Modification of Lowry's method for collagen concentration measurement. *Journal of Biochemical and Biophysical Methods, 32*(1), 33–43.

Lehninger, A., Nelson, D. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry*. New York: W.H. Freeman.

Lin, Y. K., & Liu, D. C. (2006). Comparison of physical–chemical properties of type I collagen from different species. *Food Chemistry, 99*, 244–251.

Lin, Y. K., & Liu, D. C. (2007). Studies of novel hyaluronic acid-collagen sponge materials composed of two different species of type I collagen. *Journal of Biomaterials Applications, 21*(3), 265–281.

Marotta, M., & Martino, G. (1985). Sensitive spectrophotometric method for the quantitative estimation of collagen. *Analytical Biochemistry, 150*(1), 86–90.

Nayaka, M. A. H., Sathisha, U. V., Manohar, M. P., Chandrashekar, K. B., & Dharmesh, S. M. (2009). Cytoprotective and antioxidant activity studies of jaggery sugar. *Food Chemistry, 115*(1), 113–118.

Pachence, J. M. (1996). Collagen-based devices for soft tissue repair. *Journal of Biomedical Materials Research, 33*(1), 35–40.

Qiao, H., Bell, J., Juliao, S., Li, L., & May, J. M. (2009). Ascorbic acid uptake and regulation of type I collagen synthesis in cultured vascular smooth muscle cells. *Journal of Vascular Research, 46*(1), 15–24.

Quasnichka, H. L., Tarlton, J. F., Anderson-Mackenzie, J. M., Billingham, M. E., Bailey, A. J., & Pickford, A. R. (2005). Development of an assay for the quantification of type I collagen synthesis in the guinea pig. *Journal of Immunological Methods, 297*(1–2), 133–141.

Ravelojaona, V., Robert, L., & Robert, A. M. (2008). Effect of cellular aging on collagen biosynthesis: II. Collagen synthesis and deposition by a human skin fibroblast strain over 25 passages. *Archives of Gerontology and Geriatrics, 47*(3), 368–376.

Reddy, G. K., & Enwemeka, C. S. (1996). A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical Biochemistry, 29*(3), 225–229.

Ricard-Blum, S., & Ruggiero, F. (2005). The collagen superfamily: From the extracellular matrix to the cell membrane. *Pathologie Biologie (Paris), 53*(7), 430–442.

Shahdadfar, A., Loken, S., Dahl, J. A., Tunheim, S. H., Collas, P., Reinholt, F. P., et al. (2008). Persistence of collagen type II synthesis and secretion in rapidly proliferating human articular chondrocytes in vitro. *Tissue Engineering Part A, 14*(12), 1999–2007.

Shigemura, Y., Iwai, K., Morimatsu, F., Iwamoto, T., Mori, T., Oda, C., et al. (2009). Effect of prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin. *Journal of Agricultural and Food Chemistry, 57*(2), 444–449.

Tredget, E. E., Falk, N., Scott, P. G., Hogg, A. M., & Burke, J. F. (1990). Determination of 4-hydroxyproline in collagen by gas chromatography/mass spectrometry. *Analytical Biochemistry, 190*(2), 259–265.

Tullberg-Reinert, H., & Jundt, G. (1999). In situ measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: Effects of transforming growth factor beta 2 and ascorbic acid 2-phosphate. *Histochemistry and Cell Biology, 112*(4), 271–276.

Walsh, B. J., Thornton, S. C., Penny, R., & Breit, S. N. (1992). Microplate reader-based quantitation of collagens. *Analytical Biochemistry, 203*(2), 187–190.

Williams, E. J., Benyon, R. C., Trim, N., Hadwin, R., Grove, B. H., Arthur, M. J., et al. (2001). Relaxin inhibits effective collagen deposition by cultured hepatic stellate cells and decreases rat liver fibrosis in vivo. *Gut, 49*(4), 577–583.

Woessner, J. F. Jr., (1961). The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Archives of Biochemistry and Biophysics, 93*, 440–447.