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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