Type II collagen quantification in experimental chondrogenesis

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Summary

Type II collagen is an excellent indicator of the cartilage phenotype. Accurate quantification with existing methods requires about 100 μg of collagen. We have developed a method which allows for accurate quantification of type II collagen in samples as small as 1 μg (and sample volumes as little as 1 μl). Types I and II collagen were pepsin purified, cleaved with cyanogen bromide, and dissolved in sample buffer at concentrations of 0.125–200 μg/μl. Volumes of 1 μl were analyzed by electrophoresis on microgels. The gels were scanned on a laser densitometer and the ratios of the αI(II)CB10 to the αI(II)CB7,8 plus αI(II)CB11 determined. The cyanogen bromide-derived (CNBr) peptides could be resolved at concentrations as low as 0.25 μg/μl. The ideal working concentration for purified collagens was 1–8 μg/μl. Standard mixtures of both purified and non-purified types I and II collagens were analyzed. At a concentration of 1 μg/μl the ratio of the bands referred to above was closely related to the relative proportion of type II collagen, in a polynomial fashion. At 8 μg/μl there was an almost perfect linear relationship. The presence of 15–30% type III collagen had < 5% effect on the measurements of type II collagen. The method is simple, reliable, fast and automated. It should have good potential for application in cartilage research as it permits quantitation of type II collagen in extremely small samples of tissue.

Key words: Articular cartilage, Collagen, Electrophoresis, Proteins.

Introduction

REPAIR or regeneration of articular cartilage is a topic of great interest. Studies in this area involve both in vivo and in vitro experiments [1–5]. It is generally considered that type II collagen represents an excellent indicator of the cartilage phenotype [1, 6–8]. Type I collagen, the predominant collagen in fibrous and other dense connective tissues, is often present in significant amounts in the repair tissue in healing joints, whereas normal cartilage contains only type II and other minor collagens [9–11]. Methods for type II collagen quantification that exist to date are useful for samples that contain approximately 100 μg of collagen or more [8, 12, 13]. Separation of cyanogen bromide-derived (CNBr) peptides of collagens, on sodium dodecyl sulfate (SDS)-polyacrylamide gels, by electrophoresis is perhaps the most reliable way of distinguishing types I and II collagen [14]. However, this method is labor intensive and time consuming. It would be desirable to be able to analyze much smaller samples of tissue. This becomes of great importance with in vitro studies where the amount of tissue available can be limited to as little as a few micrograms. It would also be useful if the methods could be automated and faster.

Microgels are now being used to resolve peptides in extremely small samples [15–19]. They are capable of being processed in a semi-automated manner unlike traditional SDS-polyacrylamide gel electrophoresis (PAGE) which requires a great deal of time, effort and skill to obtain consistently good results. They have been shown to be capable of separating and resolving the alpha chains of collagen though we were unable to find any reports confirming its usefulness for separating CNBr peptides.

The purpose of this investigation was to adapt the technological advantages of microgels to the existing methodology for type II collagen quantification that uses the traditional gradient PAGE system of Laemmli [20] as modified by us previously for the resolution of CNBr peptides of type I and II collagen [8]. This method does not require initial purification of the collagen and, therefore, minimizes the potential for sample loss.

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Materials and methods
PURIFICATION OF COLLAGEN STANDARDS

Purified types I and II collagen were prepared from rabbit Achilles' tendon and articular cartilage, respectively [8, 9, 21]. The tissues were diced with a scalpel and continuously stirred in a solution of 4 M guanidine-HCl in 0.05 M sodium acetate (pH 5.8) at 4°C for 24 h to remove proteoglycans [8, 22]. They were then centrifuged for 30 min at 30 000 g, the supernatant discarded, and the residue washed three times with 0.5 M acetic acid. Pepsin digestion was then performed at a concentration of 10 mg/ml (wet weight of collagen residue) in a solution containing 1 mg/ml pepsin in 0.5 M acetic acid [23]. This was stirred for 3 days at 4°C. Undigested noncollagenous proteins were removed by centrifugation at 30 000 g for 30 min. The pepsin-solubilized collagen in the supernatant was precipitated by adding NaCl to a final concentration of 2 M and stirring the solution at 4°C overnight [24]. The suspended collagen was collected by ultracentrifugation at 41 000 g for 45 min, dialyzed against 0.1 M acetic acid for 48 h at 4°C, to remove the salt, and dried in a SpeedVac (SC200, Savant Instruments, Inc., Farmingdale, NY, U.S.A.).

CYANOGEN BROMIDE CLEAVAGE

Cyanogen bromide cleavage was performed by adding the lyophilized samples to a 5% (w/v) solution of cyanogen bromide in 70% (v/v) formic acid at a ratio of 1 mg dry weight/ml formic acid [8, 13]. In the case of the nonpurified samples, a ratio of 10 mg tissue wet weight/ml formic acid was used. The solutions were de-aerated by bubbling in nitrogen gas and then the tubes were sealed and left to stand for 20–28 h at room temperature. The process was arrested by dilution with distilled water (10 ml/ml of solution) and the samples were dried in a SpeedVac. In the case of individual samples it was found ideal to work with small samples and sample volumes, such as 1 ml, which facilitates drying on the SpeedVac.

SDS-PAGE USING MICROGELS

The dried samples were dissolved in sample buffer at a concentration of 200 µg dry weight/µl of sample buffer as described previously [8, 20]. By serial dilution samples of varying concentrations were prepared for loading on the gels. The sample buffer contained 0.063 M Tris–HCl (pH 6.8), 3.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (v/v) bromophenol blue. Solubilization of the dried samples into sample buffer was promoted by agitation of the mixture in a water-bath at 60°C for 1 h. Mixtures were centrifuged to remove any debris.

Gradient and homogeneous polyacrylamide gels of various acrylamide concentrations were used to determine which gels would permit the best resolution of the peptide bands of interest. In trial studies it was determined that 20% polyacrylamide homogeneous gels yielded optimal resolution of the peptide bands of interest. These gels are commercially available in pre-cast form and were provided for the purpose of this study by Pharmacia (Pharmacia LKB Biotechnology, Uppsala, Sweden). They measure 4.3 x 5 cm and are 0.45 mm thick. This extreme thinness permits surface-loading rather than end-loading and therefore reduces the need for a complex geometry in the stacking gel and also permits higher electrical voltages to be used for more rapid separation because of improved cooling efficiency. The buffer strips are a solid gel rather than a liquid, which eliminates the need for fluids and greatly facilitates the handling. They contain 2% agarose, 0.2 M Tris-HCl, 0.2 M N-tris(hydroxymethyl)methyl-glycine (TRICINE), pH 8.1 and 0.55% SDS. The gels are placed horizontally in the Phast apparatus (Pharmacia LKB Biotechnology) and buffer strips are positioned beneath electrodes at both ends of the gel. Once the electrodes are resting on the buffer strips the samples are loaded on the gels in the following manner. A sheet of Parafilm is pressed on a plastic template which creates eight precisely spaced circular indentations in the Parafilm. A small volume (1–4 µl) of eight different samples is then placed on each of the indentations with a micropipette and a specially designed 'plastic comb', with eight equally spaced prongs corresponding to the eight indentations on the Parafilm, is touched down onto the eight droplets which results in the loading of 1 µl of each sample onto the comb by capillary action. This comb, containing equal amounts of eight different samples, sits on an arm that is lowered onto the gel to directly load the 1 µl of sample onto the surface of the stacking gel portion of the gel. The gel is then run at pre-determined settings. A three step separation program described in Table 1 was used followed by a 10-step staining/destaining program using PhastGel Blue R as the stain described in Table II (Fig. 1).

LASER DENSITOMETRY

The gels were scanned on a LKB2222-020 Ultro-Scan-XL (Enhanced Laser Densitometer, Electrophoresis Division, LKB Produkter AB, Bromma, Sweden). They do not have to be dried for this
procedure. However, for convenience of handling and for storage the gels were permitted to air dry; this can be sped up by exposing them to warm air using a hair dryer or similar device. Gel distortion does not occur because of the polyester backing. Each lane of peptides was scanned using the SCAN option of the Gel Scan XL program from Pharmacia. The peak width was set at 1 and integration was performed by the signal technique (drop line technique) using the valley automated background subtraction method. The selection of peaks was confirmed by diagramatic feedback on the monitor and manual adjustment of these automatic settings performed when occasionally needed due to incorrect peak selection. The peaks that were integrated were those corresponding to the \( \alpha 1(II)CB10 \) band, the \( \alpha 1(I)CB7,8 \) and \( \alpha 1(II)CB11 \) bands(8). The former \( (CB10 \) band) is specific for type II collagen whereas the latter includes the peptide bands from both type I \( (CB7,8) \) and type II \( (CB11) \) collagen that comigrate \( [8, 13, 25-28] \). From here on these bands will be referred to as the CB10, the CB7,8 and the CB11 bands, respectively.

**TEST CONDITIONS**

As stated above, a variety of different gels were tried in an attempt to reproduce the accuracy and resolution reported previously for the traditional Laemmli method \( [8, 20] \).

To determine the optimum sample concentration, a series of gels were run using sample concentrations ranging for 200 to 0.125 \( \mu g/\mu l \) in serial dilutions. The bands were able to be resolved at concentrations as low as 0.25 \( \mu g/\mu l \). Concentrations >64 \( \mu g/\mu l \) caused some distortion of the peptide bands on the gel. The ideal concentration range appeared to be from 1–8 \( \mu g/\mu l \).

A series of 10 gels were loaded with standard mixtures of purified type I and II collagen peptides in the ratio of 0, 20, 40, 50, 60, 80 and 100% type II collagen. For each mixture a volume of 1 \( \mu l \) of sample buffer containing 1 \( \mu g/\mu l \) of collagen was loaded onto a lane of the gel. These peptides were separated, stained, destained and integrated as described in Tables I and II.

An additional 11 gels were run with identical mixtures of type I and II collagenous tissues that were nonpurified (i.e. not GuHCl extracted, pepsin digested or salt precipitated) but obtained from the same sources (Achilles’ tendon and articular cartilage). These were loaded in concentrations of 8 \( \mu g/\mu l \) wet weight. It is preferable to employ wet weights rather than dry weights in experimental studies because desiccation increases the likelihood of experimental error \( [8] \).

The possible effect of the presence of type III collagen on quantification of type II collagen in such samples was evaluated. The same standard mixtures of types I and II collagen (after cyanogen bromide digestion) used and referred to above were combined with purified type III collagen (that had also been digested in cyanogen bromide) in mixtures so that the final concentrations of type III collagen was 0, 15 or 30% in each of the standardized mixtures of type I and II. A total of 32 samples were studied. The amounts of type III collagen, 15 and 30%, would represent reasonable approximations

### Table I

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Fig. 1. Serial dilutions from 0.125 μg/μl to 200 μg/μl of purified type II collagen were electrophoresed to determine the potential and optimum concentrations of collagen that could be measured. Resolution was possible down to 0.25 μg/μl. Distortion was a problem above 64 μg/μl. Reproducibility was excellent in the range of 1-8 μg/μl and good up to 32 μg/μl.

for the expected amounts of type III collagen in immature and newly formed tissue [29–31].

STATISTICAL ANALYSIS

Linear and polynomial regression analyses were used to determine the lines of best fit.

Results

In the initial studies examining different concentrations of sample loaded onto the gel, the peptide ratios able to be resolved were concentrations as low as 0.25 μg/μl. Distortion was a problem above 64 μg/μl. Reproducibility was excellent in the range of 1-8 μg/μl and good up to 32 μg/μl (Fig. 1).

Regression analyses of the ratio of CB10 divided by the (CB11 + CB7,8) bands for the purified collagen mixture was best fitted with a polynomial curve with a highly statistically significant relationship between the ratio and the percentage of type II collagen in the mixtures ($R^2 = 0.998; P = 0.0002$; Fig. 2).

Fig. 2. A typical gel showing the electrophoretic patterns of a standard set of mixtures of purified types I and II collagen from 0 to 100% type II collagen. The total quantity of collagen loaded in each lane was 1 μg (1 μl of 1 μg/μl). The α(II)CB10 band (labeled CB10) is specific for type II collagen. The α(II)CB11 band of type II collagen comigrates with the α(I)CB7&8 bands of type I collagen. The ratio of the CB10/(CB11 & CB7,8) bands correlates with the percentage of type II collagen in the mixture.
The standard mixtures of nonpurified types I and II collagen run at 8 µg/µl revealed a perfect linear relationship between the ratio and the percentages of type II collagen in the mixtures \((R^2 = 0.99; P = 0.0001; \text{Figs 3 and 4})\).

\[
\text{Ratio} = \frac{\text{CB10}}{\text{CB7,8 + CB11}} = 0.01(\% \text{ type II}) + 0.04
\]

This relationship is almost perfectly linear with a slope of 1 which greatly simplifies the analyses because the percentage of type II collagen in the sample is, therefore, equal to the ratio itself. No complex calculations are required.

The percentage of 15 or 30% type III collagen in these standard mixtures had <5% effect on the measurements of type II collagen. The slope and intercepts of the regression lines for the standard mixtures of types I and II collagen were unaffected by type III.

**Discussion**

This method of quantification of type II collagen is simple, reliable and accurate. It is also extremely fast. Most importantly, it provides a technique for accurate analysis of extremely small samples that are obtained either in vitro or in vivo. The methodology is based on techniques that have been well worked out using traditional time-consuming SDS-PAGE systems but which have been miniaturized, automated, computerized and greatly sped up. It appears to maintain the advantages of the
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traditional methods while eliminating many of their disadvantages. The advantages of this system can be considered individually.

ANALYSIS OF EXTREMELY SMALL SAMPLES

A very important contribution of this method is that extremely small samples in the range of 1 μg of collagen and only 1 μl of sample can be analyzed. This represents approximately 100 times less tissue than is required for the method from which this technique has been modified. The collagen in the samples does not have to be purified initially or digested with pepsin and therefore the total number of steps involved is greatly reduced and the potential for sample loss or experimental error are markedly diminished. Type II collagen quantification is, thus, possible with very small samples obtained from experiments that would otherwise not be able to be analyzed. Indeed, this methodology allows in vitro experiments to be performed with very small explants, thereby greatly decreasing the costs for experimental animals, chemicals, etc.

TIME SAVINGS

For a number of reasons a great deal of time is saved with this method. First, the collagens in the sample do not have to be purified or digested with pepsin, which normally takes 3–4 days. Second, since very small samples are used, they can be dried quickly after CNBr cleavage in large numbers in a SpeedVac system. Typically, 150 samples can be dried in 4–6 h. Third, since the gels are available commercially at a reasonable cost, no time or training is required for their preparation. Another day is saved in this step. The microsized gels permit greatly reduced elution, staining and destaining times as well as drying time. Total processing time for running, staining and destaining the gel is only 2 h and two gels can be run simultaneously. Furthermore, one pair of gels can be running in the electrophoresis unit while another pair of gels is in the staining/destaining unit. Thus, the actual turn-around time is approximately 1.5 h permitting as many as 12 gels to be run in one day. Finally, the use of a laser densitometer, requires minimal time to operate. All the steps are computerized and the data stored automatically.

SIMPLICITY

Gels are mounted on a polyester backing which makes them physically quite easy to handle and protects the gels from distortion during staining, destaining or drying. They can be stored in regular glass slide mounts which permits their projection on a slide projector. They are simply air-dried and do not require the use of special gel dryer.

Another element of simplicity is the fact that all the chemicals required for the staining/destaining are drawn in and expelled automatically through fine tubes connected to storage bottles for each solution. Minimal handling of materials is required. Traditional pouring of gradient gels requires exposure to the neurotoxin acrylamide. This is not necessary with pre-cast gels.

REPRODUCIBILITY

The use of pre-cast gels and automation greatly reduces the number of factors that vary from one experiment to another. Also, because of the automation, the system does not require careful observation by a technician for the duration of processing at any step.

In summary, an accurate, reliable, simple, fast and efficient method for quantification of type II collagen in small samples (1 μg) has been presented. The advantages of this method are many. It represents an adaption of modern sophisticated technology to time-honored techniques for collagen typing. The applicability of this method to the field of cartilage research, where the ratio of type I to type II collagen is an important indicator of phenotypic expression and where available samples are typically too small for traditional methods of quantitative collagen analysis, is readily apparent.

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References


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