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REGULAR ARTICLE

Quantitative analysis of denatured collagen by collagenase digestion and subsequent MALDI-TOF mass spectrometry

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Abstract Collagens are the most abundant proteins in vertebrate tissues and constitute significant moieties of the extracellular matrix (ECM). The determination of the collagen content is of relevance not only in the field of native tissue research, but also regarding the quality assessment of bioengineered tissues. Here, we describe a quantitative method to assess small amounts of collagen based on MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry (MS) subsequent to digestion of collagen with clostridial collagenase (clostridiopeptidase A) in order to obtain characteristic oligopeptides. Among the resulting peptides, Gly-Pro-Hyp, which is highly indicative of collagen, has been used to assess the amount of collagen by comparing the Gly-Pro-Hyp peak intensities with the intensities of a spiked tripeptide (Arg-Gly-Asp). The approach presented herein is both simple and convenient and allows the determination of collagen in microgram quantities. In tissue samples such as cartilage, the actual collagen content has additionally been determined for comparative purposes by nuclear magnetic resonance spectroscopy subsequent to acidic hydrolysis. Both methods give consistent data within an experimental error of ±10%. Although the differentiation of the different collagen types cannot be achieved by this approach, the overall collagen contents of tissues can be easily determined.

Keywords MALDI-TOF mass spectrometry · Extracellular matrix · Collagen · Cartilage · Gelatine · Collagenase · Quantitative data analysis

Ariane Nimptsch and Stephanie Schibur contributed equally to this work.

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Introduction

Collagens are the most abundant proteins on earth and comprise about 25%-30% of the proteins in vertebrate organisms (Brinckmann 2005; Gordon and Hahn 2010). The collagen superfamily is steadily growing, and at least 28 different collagen types encoded by more than 40 genes are currently known that function as essential structural components in many tissues (Eyre 2004). Collagens are primarily located in the extracellular matrix (ECM) of skin, tendon, bone, cartilage, and many other tissues (Cremer et al. 1998). With a tensile strength comparable with that of steel, fibrillar collagen represents an ideal material to impart stability to these various tissue types (Huster 2008 and references cited therein).

In the articular cartilage of the joints, the relevance of which will be discussed below in more detail, collagen types II, IX, and XI are primarily found with smaller contributions of types III, VI, VII, and XIV (Eyre 2002). Collagen type II (a fibrillar type) is most abundant, whereas type IX is a fibril-associated collagen that is of significance because it mediates binding to proteoglycans (PG). The sheet-forming collagen type X is only expressed by

hypertrophic chondrocytes and is involved in the organization of the collagen type II network (for a more detailed survey of the collagens of cartilage, see Responte et al. 2007).

Collagen can be regarded as the counterpart of the PG in articular cartilage (Schiller et al. 2006). The carbohydrate constituents of PG, the glycosaminoglycans (GAG), such as hyaluronan, keratan-, and chondroitin sulfate, are highly polar because of their negative charges (sulfate and carboxylate groups) and tend to absorb large quantities of water leading to the pronounced swelling of the ECM. On the other hand, the intact collagen network limits the swelling of GAG and reduces the water absorption of cartilage. Therefore, an appropriate ratio between collagen and GAG is crucial in order to warrant the excellent mechanical properties of cartilage (Martel-Pelletier et al. 2008).

Because of the extreme incidence of rheumatic diseases, particularly those such as rheumatoid arthritis or osteoarthritis (Schiller et al. 2003), and sport injuries and the related socioeconomic consequences, the cure of cartilage defects is of considerable interest. Unfortunately, the regenerative capacity of articular cartilage is limited, and degenerated cartilage can only be replaced by the organism itself to an extremely limited extent.

Therefore, the transplantation of healthy cartilage cells, chondrocytes, or stem cells that can be differentiated into chondrocytes is regarded as a promising method for the treatment of cartilage lesions (Kuo et al. 2006). However, the ECM produced by these cells usually does not exhibit the excellent mechanical properties of the native articular cartilage tissue. Among other factors, bioengineered cartilage is characterized by an increased GAG and decreased collagen content (Schulz et al. 2006; Haberhauer et al. 2008). Thus, in order to improve the quality of bioengineered cartilage by optimizing the culture conditions, the determination of (at least) the relative collagen contents in cartilage explants is imperative. As not all collagen types are capable of forming fibrils, which is crucial for mechanical stability (Eyre 2002), the differentiation of the individual collagen types also becomes an important task.

The determination of collagen is mostly based on chromatographic techniques (Deyl et al. 2003), electrophoresis, or capillary electrophoresis (Deyl and Miksík 1995) and immunoblotting (Rucklidge et al. 1997), whereas spectroscopic techniques such as nuclear magnetic resonance (NMR) have been employed only in a few cases (Huster et al. 2002), despite the advantage that the concomitant determination of collagen and GAG is possible in native tissues. Unfortunately, the majority of spectroscopic techniques are of low sensitivity and only applicable if the collagen-containing sample is available in significant amounts (at least 100 μg).

With the development of the soft ionization mass spectrometry (MS) methods, viz., ESI (Fenn et al. 1989) and MALDI (Karas and Hillenkamp 1988), highly sensitive analytical tools have become available (Baldwin 2005). According to current knowledge, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) seems particularly suitable for the direct detection of proteins and peptides in biological samples, because it is highly sensitive and robust with regard to sample impurities such as salts and detergents (Fuchs et al. 2008). Thus, MALDI-TOF-MS has previously been successfully applied to the qualitative analysis of collagens: fibril-forming collagens are composed of linear alpha chains of about 100 kDa (i.e., about 1000 amino acid residues), which form closely coiled triple-stranded helical macromolecules. The detection of type III chain proteins from calf skin by employing ultraviolet MALDI-MS and sinapinic acid as the matrix has been reported (Kim et al. 2000), but this approach confers several limitations: the intact collagen triple helix is not detected, and the individual chains are only detectable after thermal denaturation of the sample (Hambleton and Shakespeare 1991).

In contrast, the analysis of native collagen, even that of the non-covalently bound triple helices, is facilitated if infrared (IR) instead of UV-MALDI-MS is used. For instance, chicken collagen of types I, II, and XI have been identified by IR-MALDI-MS by using succinic acid as the matrix (Zaia et al. 1997), whereas Dreisewerd and coworkers have identified whole fibril-forming collagen proteins of types I, III, and V in fetal calf skin by using glycerol as the matrix (Dreisewerd et al. 2004). The use of careful and sophisticated sample preparation has allowed even the high-mass collagen type III of several hundred kiloDaltons to be detected as an intact molecule (Dreisewerd et al. 2004).

Although the detection of intact collagen is basically possible, the reproducibility of protein mass spectra of high masses (>100 kDa) has proven poor so far and therefore, to the best of our knowledge, not suitable for the (at least semi-quantitative) evaluation of the collagen contents of biological samples. Consequently, Henkel and Dreisewerd (2007) have performed the cyanogen bromide (CNBr) degradation of the fibrillar collagens I, III, and V prior to MS analysis and have been able to identify distinct peptides that serve as reference signals of the individual α chains. Additionally, peptide glycosylation and the cross-linking of peptides can also be monitored, although no quantitative data regarding the collagen contents of tissues can be obtained.

Unfortunately, the most abundant collagen type II of cartilage (Eyre et al. 2006) has been little investigated by MS methods to date, and as far as we know, only a single reference is available, viz., that by Zhang et al. (2006).

These authors have demonstrated that characteristic fragments enable the differentiation between collagen types I and II by using isolated bovine collagen and cartilage subsequent to thermal denaturation and tryptic digestion. Nevertheless, many open questions remain (Bornstein and Traub 1979), particularly regarding a potential quantitative evaluation of the obtained mass spectra (Tsikas 2010).

Therefore, the aim of this paper is to show that bacterial collagenase can be used to digest native collagen, and that the obtained characteristic tripeptides can be quantitatively analyzed by standard UV-MALDI-TOF-MS. However, a differentiation of the individual collagen types is not possible by this approach. Thus, only an overall determination of the total amount of collagen is possible. In addition to gelatine as a simple example, the collagen content of a selected cartilage sample will also be investigated in order to verify the suitability of this new method to tissue samples. Data are compared with ^{1}H NMR compositional data subsequent to acidic hydrolysis.

Materials and methods

Chemicals

Unless otherwise stated, the chemicals (salts, buffer components, and MALDI matrices), oligopeptides, and (deuterated) solvents used were obtained at highest commercially available purity from Sigma-Aldrich (Taufkirchen, Germany). This also holds for the employed collagen (bovine collagen type I), the gelatine (from porcine skin), the collagenase A (EC 3.4.24.3 from *Clostridium histolyticum*), chondroitin sulfate, and standards (Arg-Gly-Asp and 3-trimethylsilyl-propionate-d_4 [TSP]). The matrix 9-aminoacridine (9-AA) was obtained from Acros Organics (Moris Plains, N.J., USA).

MALDI-TOF mass spectrometry

All MALDI-TOF mass spectra were acquired on an Autoflex I mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a 337-nm pulsed nitrogen laser. All measurements (positive and negative ion detection) were performed in the reflector mode by using delayed extraction conditions. The extraction voltage was 20 kV, and gated matrix suppression was applied to prevent detector saturation by matrix ions with *m/z* lower than 200 (Fuchs et al. 2008). One hundred single laser shots were averaged for each mass spectrum, whereby the laser was randomly moved over the sample in order to average analyte/matrix inhomogeneities: the achievable level of detection (LOD) of MALDI-TOF-MS for a specific compound has been shown to be mainly limited by the level of chemical background noise (Rohlfing et al. 2007). Therefore, the use of a larger number of laser shots does not significantly improve spectral quality.

2,5-Dihydroxybenzoic acid (DHB) as a 0.5 M solution in methanol containing 0.1% trifluoroacetic acid (TFA) was exclusively used for positive ion detection, whereas 9-AA (10 mg/ml in acetonitrile/isopropanol [40/60, v/v]) was used as the matrix for negative ion detection. All samples contained a known amount of Arg-Gly-Asp as the internal standard and were mixed (1:1, v/v) with the matrix prior to application to the target. Although more sophisticated sample preparation techniques (Fuchs et al. 2008) were also attempted, no major improvements could be obtained. The concentrations of the individual tripeptides and the gelatine and tissue digests are given in the Results and Discussion section. All spectra were analyzed by using FlexAnalysis (2.2) software provided by Bruker Daltonik (Bremen).

Post-source decay (PSD) experiments were also performed in selected cases to confirm peak assignments. Here, the precursor ions of interest were isolated by using a timed ion selector. The laser intensities for each first PSD fragment ion spectrum were maintained the same as those in the standard reflector-mode spectrum and were gradually enhanced for the other segment spectra. The fragment ions were refocused onto the detector by stepping the voltage applied to the reflectron in appropriate increments. This was performed automatically by using the fragment analysis and structural TOF (FAST) subroutine of the Flex Control Program (Bruker Daltonics). Further details on PSD analysis are available in (Fuchs et al. 2008).

Enzymatic digests of native collagen and gelatine

Individual collagen (1 mg) and gelatine (1 mg) samples were suspended in 200 µl 50 mM TES (N-[TRIS (hydroxymethyl)methyl]-2-aminoethane sulfonic acid) buffer containing 0.36 mM $CaCl_2$ at pH 7.4 to give a concentration of 5 mg/ml. Some residual water bound to the used biopolymers was not further considered, but this problem will be discussed below in more detail. Complete dissolution of the samples was checked by visual inspection for the presence of residual solid material. After heat denaturation (10 min at 95°C), 4 µl collagenase (0.1 mg/ml) was added before digestion was performed at 37°C for 24 h. No major efforts were made to control carefully the activity of the enzyme, because complete digestion was the only requirement of this assay. This could be easily accomplished by the addition of a sufficient excess of collagenase. During the experiments, various preparations of collagenase I (clostridiopeptidase) were used, but all gave similar results. Thus, the type of the used collagenase was not important. The completeness of the digestion was

checked by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by ^{1}H NMR spectroscopy. Subsequently, 5 μl collagenase digests were each mixed with 2.5 μl Arg-Gly-Asp tripeptide (0.25 mg/ml). Thus, the most concentrated solution contained 3.33 mg/ml and 0.083 mg/ml Arg-Gly-Asp tripeptide, respectively, as an internal standard. The concentration of this standard was kept constant in all cases. The sample was then mixed with the matrix solution (7.5 μl) and subsequently analyzed by MALDI-TOF-MS. Collagen and gelatine stock solutions were diluted as appropriate. The applied volume that was deposited onto the MALDI target was always 1 μl.

Collagen content estimation by ^{1}H NMR spectroscopy

These investigations were performed in analogy with a previously performed study (Schiller et al. 2001). Fresh porcine articular cartilage was obtained from a healthy juvenile animal. After separation from the bone, the cartilage (50 mg) was cut into small pieces and digested by collagenase (1 ml TES+100 μl collagenase, 0.1 mg/ml), and the digests were investigated by MALDI-TOF MS as described above. Notably, higher amounts of tissue samples than pure collagen were required because of the considerable water content of cartilage. As the absolute collagen content of the cartilage as such was unknown, a small amount of cartilage (10 mg) was hydrolyzed by treatment with 6 M HCl (1 ml), a commonly accepted method to obtain the individual amino acids (Torchia et al. 1977 and references cited therein). For the sake of comparison, a gelatine sample (10 mg) was also prepared in 6 M HCl (1 ml). The hydroxyproline content was not determined by MS but by high resolution NMR spectroscopy, because NMR spectra can be recorded directly in the presence of 6 M HCl, and no additional sample purification is required.

All NMR measurements were performed on a Bruker AVANCE-600 spectrometer operating at 600.13 MHz for ^{1}H. All spectra were recorded at 37°C (310 K) by using a "direct" broadband NMR probe. Typically, 0.50 ml samples from the HCl incubation experiments of gelatine and cartilage and 100 μl of a 1.5 mg/ml (8.71 mM) 3-trimethylsilyl-propionate (TSP) solution were placed in a 5-mm NMR tube. Thus, the final concentration was 8.3 mg/ml. Since the volume was 600 μl, this corresponded to 5 mg absolute amount of cartilage within the NMR tube. The residual water signal (HDO) was suppressed by presaturation on the water resonance frequency. Transients (n=128) were acquired for each spectrum by using a repetition time of 5 s. All spectra were recorded with a spectral width of 6000 Hz (10 ppm) and 32 k data points. No window functions were used prior to Fourier transformation (LB=0).

Chemical shifts were referenced to the trimethylsilyl resonance of TSP, and this compound was also used in order to determine the concentrations of the relevant amino acids. Spectra of amino acid mixtures (glycine and 4-hydroxyproline, each at 5 mg/ml final concentration in the NMR tube) were also recorded in 6 M HCl for comparative purposes.

Results and discussion

Proteolytic cleavages with specific proteases such as trypsin or endoproteinase AspN are commonly used for proteomics applications in order to obtain characteristic fragments of the protein of interest (Thiede et al. 2005; Aebersold and Mann 2003). However, the insolubility of native intact collagen represents a significant problem because the majority of proteases used for proteomic applications digest only soluble proteins but do not cleave native collagen.

Therefore, the use of bacterial collagenases can be regarded as a straightforward approach because these enzymes also fragment native helical collagen. As Clostridial collagenases are readily available in large amounts and inexpensive, they are, on the one hand, the enzymes of choice to digest native collagen into smaller peptides that are subsequently easily detectable by MS. On the other hand, bacterial collagenases have the disadvantage that the collagen is fragmented rather unspecifically into small peptides leaving no information concerning collagen type: The preferred cleavage site of collagenase from *Clostridium histolyticum* is between ~Pro-X and Gly-Pro-Y, whereby "X" is most often a neutral amino acid and "Y" can be any non-specific amino acid residue. Additionally, compounds containing hydroxyproline (Hyp) instead of proline are also affected (Bond and Van Wart 1984). Therefore, small peptides composed of only a few amino acids are expected to be generated upon collagenase digestion.

In this study, we have used a MALDI-TOF device, although the dynamic range of the TOF mass analyzer is limited and covers (particularly in the smaller mass range) only about one order of magnitude. The use of MALDI-TOF has been motivated by the fact that the majority of protein scientists have access to a MALDI-TOF MS device, and thus, our protocol is widely applicable.

One drawback of MALDI-MS regarding the detection of smaller molecules is the interference of small analyte molecules with matrix ions, as the matrix is present in vast excess over the analyte. From our experience, 2,5-dihydroxybenzoic acid (DHB) is the matrix of choice for positive ion MALDI-MS as it provides far fewer signals compared with cinnamic-acid-derived matrices (Schiller et al. 2007).

In order to evaluate the potential overlap between collagen-derived peptides and matrix signals in more detail, diverse synthetic peptides were initially investigated. The obtained MALDI mass spectra of the selected peptides

were additionally compared with bovine collagen type I subsequent to exhaustive digestion with collagenase from *Clostridium histolyticum*. The positive ion MALDI-TOF mass spectra of selected peptides mimicking potential digestion products of collagen are shown in Fig. 1 (cf. also Table 1 for more detailed assignments).

In Fig. 1a, the trace corresponds to Gly-Pro-Ala, which is easily detected as the Na^+ and K^+ adduct at *m/z* 266.1 and 282.1. Because of the presence of the amino and carboxylic acid group, H^+/Na^+ and H^+/K^+ exchanges are also observed, and the corresponding peaks are explicitly assigned in Fig. 1. All other mass spectra of the various peptides (Gly-Pro-Thr, Fig. 1b; Gly-Pro-Hyp, Fig. 1c; Gly-Pro-Glu Fig. 1d; Gly-Pro-Arg Fig. 1e) can be explained in the same way as that for Gly-Pro-Ala (Fig. 1a) and will thus not be comprehensively discussed here. The same holds for the variations of the intensity ratios between the individual adducts, variations that presumably stem from differences in the salt content of the commercially available tripeptide samples.

The same peaks as detected particularly in traces (Fig. 1c, d) are obviously detectable in the MALDI mass spectrum recorded subsequent to the digestion of bovine collagen type I with collagenase (Fig. 1f). Some selected peaks that occur in both the spectra of the tripeptides and in the collagen digest are indicated by vertical dotted lines. We consider it reasonable to assume that Gly-Pro-Glu (Fig. 1d) and particularly Gly-Pro-Hyp (Fig. 1c) contribute most massively to the signals observed in the collagen digest, because Hyp and Glu are highly abundant amino acid residues of collagen (Eyre 2004).

An unequivocal assignment of the peaks at *m/z* 308.1 and 324.1 cannot be made based solely on their *m/z* ratios without performing additional MS/MS experiments, because of the superposition of the different adducts of the individual tripeptides. However, these peaks can evidently be used as markers of collagen. Although the contribution of peptides derived from other proteins (e.g., in tissue samples) is highly unlikely because collagen is the most abundant protein, we will explicitly show below that these peaks are also reliable measures if native cartilage instead of isolated collagen is investigated. The five tripeptides shown in Fig. 1 were also combined to give an equimolar mixture in order to investigate to what extent the individual peptides were detectable under our experimental conditions (Fig. 2).

The trace in Fig. 2a represents the positive ion MALDI-TOF mass spectrum of a 0.2 mM solution of each peptide, and this stock solution was diluted in several 1:5 dilution steps. Thus, the peptide concentration was 40 μM in Fig. 2b, 8 μM in Fig. 2c, and 1.6 μM in Fig. 2d. Considering that only 1 μl peptide solution is required for successful MALDI analysis and assuming a rough molecular weight of 300 g/mol, this means that only about 5 pg peptide is needed to record a reasonable mass spectrum (cf. Fig. 2d).

The protonated species are marked by their respective *m/z* values and indicated by vertical dotted lines, whereas the sodiated peptide species are marked by diamonds (Fig. 2). The individual peptides are evidently not detectable to the same extent, and the intensities decrease in the order Gly-Pro-Arg (*m/z* 329.2) >Gly-Pro-Ala (*m/z* 244.1) > Gly-Pro-Hyp (*m/z* 286.1) ≈ Gly-Pro-Glu (*m/z* 302.1) > Gly-Pro-Thr (*m/z* 274.1). Unsurprisingly, the most cationic tripeptide with an arginine residue is the most sensitively detectable in the positive ion mode by using an acidic matrix such as DHB. Thus, Gly-Pro-Arg is still detectable at the highest dilution. Because following digestion of collagen with collagenase, the signal of Gly-Pro-Hyp is of the highest intensity (cf. Fig. 1f), interest was paid primarily to this tripeptide (the determined signal-to-noise ratios [*S/N*] are indicated in bold italic numbers in Fig. 2). Of course, the pronounced DHB signals complicate the data analysis, for instance at *m/z* 273.0 (Schiller et al. 2007). Expectedly, the intensity of this peak increases with increasing dilution (Fig. 2, underlined numbers), whereas the intensity of Gly-Pro-Hyp (Fig. 2, bold italic numbers) decreases upon dilution. Thus, these data clearly indicate that quantitative analysis is basically possible. Although all peptides are also detectable as negative ions, particularly if a basic matrix such as 9-aminoacridine is used (data not shown), the negative ion detection mode provides (for unknown reasons to date) much lower sensitivity in comparison with the positive ion mode (Shroff et al. 2007). This is unfortunate as negative ion spectra can be more easily interpreted because of the lack of the superposition of signals of the diverse collagen adducts.

In the next step (Fig. 3), a variable amount of Gly-Pro-Hyp, which is most indicative of collagen (cf. Fig. 1), was monitored by MALDI-TOF-MS with reference to the intensity of a specific peptide standard, namely Arg-Gly-Asp, which was added in all cases at a fixed concentration (0.25 mg/ml, resulting in an absolute amount of 250 ng on the MALDI target). This reference tripeptide has been used because it possesses a higher monoisotopic mass (346.2 atomic mass units) than the relevant collagen-derived tripeptides and, thus, does not lead to interference with the analyte. Additionally, this peptide is commercially available at a moderate price. We also attempted to use other tripeptides, but no major gain of accuracy (only a loss of sensitivity) was observed.

The intensity of the H^+ adduct of Gly-Pro-Hyp, in particular, obviously decreases upon dilution. However, the contribution of the individual adducts is evidently not constant but differs between the individual peptides dependent on the dilution. Therefore, only relative quanti-

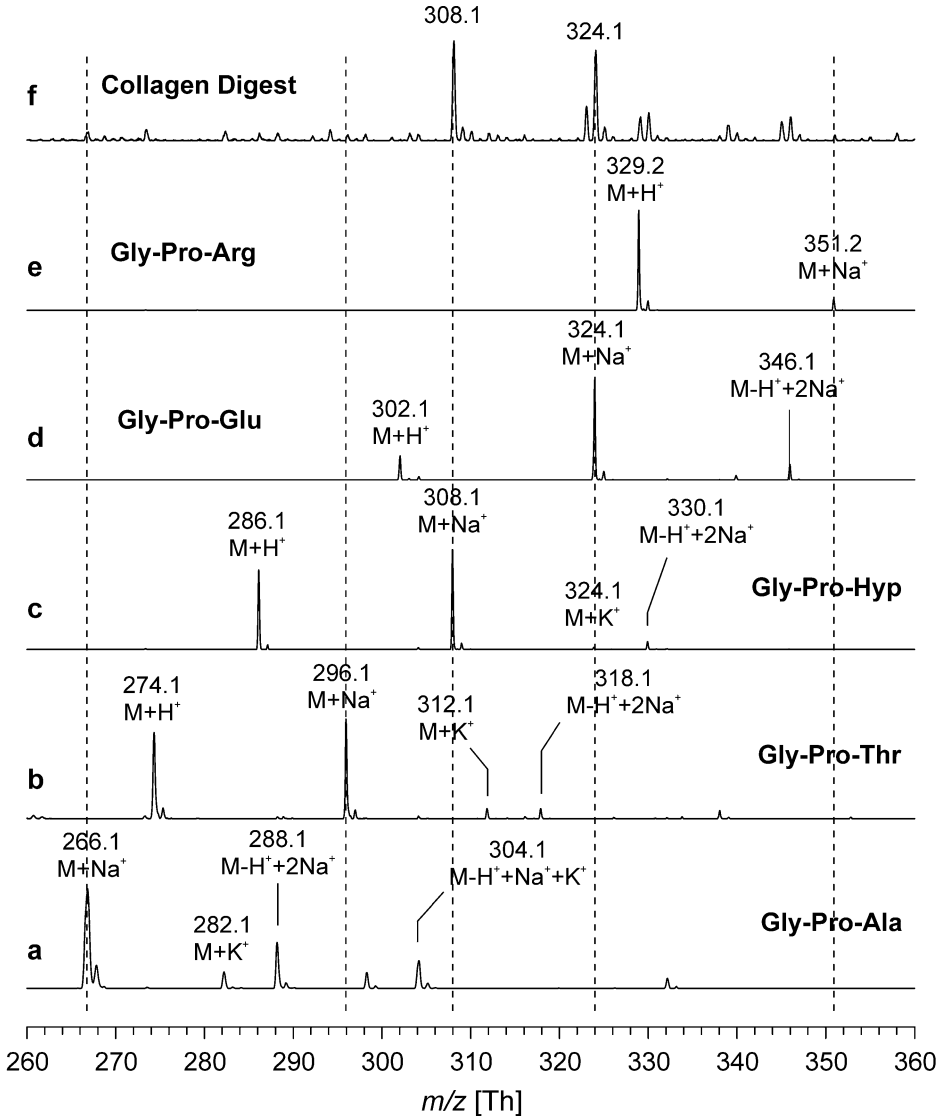

Fig. 1 Positive ion MALDI-TOF mass spectra of Gly-Pro-Ala (**a**), Gly-Pro-Thr (**b**), Gly-Pro-Hyp (**c**), Gly-Pro-Glu (**d**), and Gly-Pro-Arg (**e**). The mass spectrum of bovine collagen type I subsequent to digestion with collagenase from *Clostridium histolyticum* is shown in **f** for comparative purposes. The *vertical dotted lines* indicate peaks that are detected in the spectrum of the collagen digest and in the spectra of the model peptides. All MALDI mass spectra were recorded with DHB as matrix. Note that all spectra are scaled in a way such that the base peak possesses the same intensity. Therefore, y-axes are not provided. Quantitative data are provided in Fig. 2

fication is possible by comparing the intensities of, e.g., either H^+ or Na^+ adducts. Unfortunately, the generation of Na^+ adducts cannot be avoided, even if the spectra are recorded in the presence of TFA as an additional cationizing species. Therefore, the sum of the intensities of the sodiated and protonated species was used for further analysis. Additionally, fluctuations of the laser fluences that might favor the generation of a certain adduct play a much smaller role under these conditions.

Table 1 Formulae and monoisotopic masses (Mr) of the collagen reference peptides used. Arg-Gly-Asp represents the reference compound for quantification

Peptide	Formula	Monoisotopic mass (Mr)
Gly-Pro-Ala	$C_{10}H_{17}N_3O_4$	243.1
Gly-Pro-Thr	$C_{11}H_{19}N_3O_5$	273.1
Gly-Pro-Hyp	$C_{12}H_{19}N_3O_5$	285.1
Gly-Pro-Glu	$C_{12}H_{19}N_3O_6$	301.1
Gly-Pro-Arg	$C_{13}H_{24}N_6O_4$	328.2
Arg-Gly-Asp	$C_{12}H_{22}N_6O_6$	346.2

In Fig. 4, the intensity ratio between Arg-Gly-Asp (standard) and Gly-Pro-Hyp (analyte) is plotted against the absolute amount of Gly-Pro-Hyp on the MALDI target. The absolute amounts are given in all cases because a solid sample (but not a solution) is actually investigated by MALDI-TOF MS. However, concentrations of the original solutions can be easily calculated if the applied sample volume (1 μl) and the amount of matrix solution is known. A linear relationship evidently exists over nearly one order of magnitude and allows an assessment of the amount of Gly-Pro-Hyp. Nevertheless, significant deviations occur from linearity if lower and higher amounts of the tripeptide are investigated. This has previously been explained for the quantitative analysis of carbohydrate constituents of the ECM

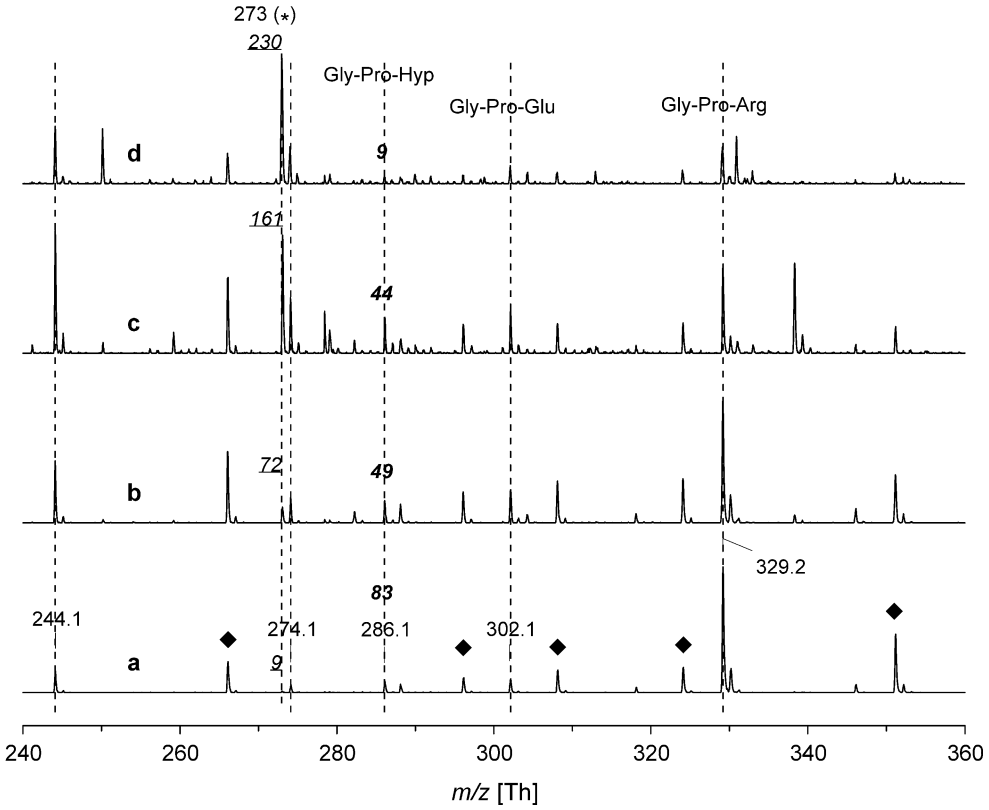

Fig. 2 Positive ion MALDI-TOF mass spectra of an equimolar mixture of Gly-Pro-Ala, Gly-Pro-Thr, Gly-Pro-Hyp, Gly-Pro-Glu, and Gly-Pro-Arg. The concentration of each peptide was 0.2 mM (**a**), 40 μM (**b**), 8 μM (**c**), and 1.6 μM (**d**). Aliquots of 1 μl of a 1:1 (v/v) mixture of the matrix and analyte was deposited onto the MALDI target. Thus, the absolute amounts on the MALDI target were 100, 20, 4, and 0.8 nmol. The protonated species are indicated by the m/z value, whereas the corresponding Na^+ adducts are marked by *diamonds*. The *vertical dotted lines* indicate protonated species of the individual peptides. All spectra were recorded with DHB as matrix. The peak at m/z 273.0 (*star*) is derived from DHB. The signal-to-noise ratios (S/N) of Gly-Pro-Hyp (*bold*) and the characteristic DHB peak at m/z=273.0 (*underlined*) are indicated. The S/N of the matrix signal obviously increases, whereas that of the tripeptide decreases upon dilution

(Nimptsch et al. 2009): if extremely small amounts of Gly-Pro-Hyp are analyzed, large intensity ratios can be expected, and thus, considerable deviations may occur. In contrast, if extremely high amounts of Gly-Pro-Hyp are analyzed, the matrix-to-analyte ratio becomes suboptimal leading to lower spectral qualities and, thus, less accurate measurements. In the worst case, complete analyte suppression may even occur. The data shown in Fig. 4 should be regarded as a type of calibration curve, and we recommend that each user collects an independent calibration data set. This is strongly advisable because detector sensitivities may change if a different MALDI-TOF mass spectrometer is used.

Although the positive ion MALDI-TOF mass spectra of isolated peptides can obviously be quantitatively analyzed, some assumptions clearly have to be made if this method is to be used to determine the collagen contents of tissue samples:

1. The collagenase digestion of the tissue must be complete, as otherwise the (absolute) collagen content of the tissue is underestimated. In our opinion, this is a minor problem, because the presence of larger peptides and residual collagen can easily be checked by MALDI MS or SDS PAGE, respectively.
2. The salt contents of the peptide solutions must be constant, or otherwise the sum of the intensities of the individual adducts of a given species must be used. This is the most reliable approach because, according to our experience, the generation of alkali metal adducts (in biological samples) can never be completely suppressed even if, for example, TFA is added as an additional cationizing agent.

In order to investigate whether this method is also applicable to the quantification of the collagen contents of native tissues, gelatine subsequent to collagenase digestion (as a simple test system) was initially investigated, and the resulting amounts of Gly-Pro-Hyp were quantified as described above. Gelatine represents denatured collagen and is therefore a simpler test system than insoluble native collagen

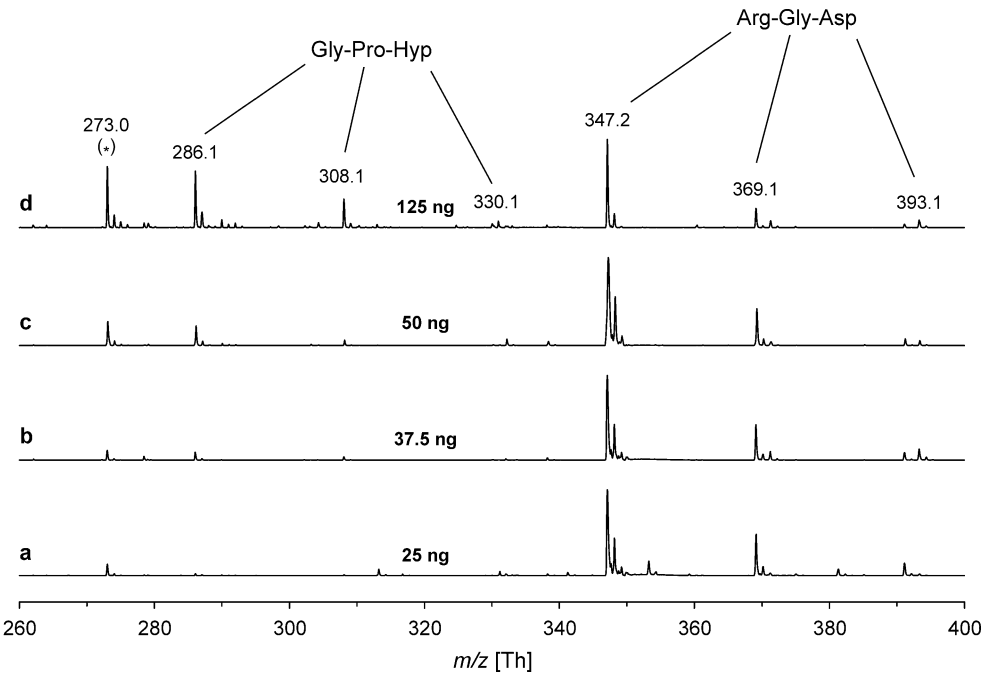

Fig. 3 Positive ion MALDI-TOF mass spectra of mixtures between Gly-Pro-Hyp and Arg-Gly-Asp. All spectra were recorded with DHB as matrix. The absolute amount of Arg-Gly-Asp was fixed (41.5 ng corresponding to 0.12 nmol on the MALDI target), whereas the absolute amount of Gly-Pro-Hyp on the MALDI target was varied as indicated by appropriately diluting the stock solutions. Peaks are marked according to the m/z ratio. The peak at m/z 273.0 (*star*) is derived from the DHB matrix. All spectra are scaled in a way that the standard (m/z 347.2) possesses the same intensity

or tissues that contain significant amounts of GAG that might affect the accuracies of the MALDI MS measurements. Nevertheless, tissue collagen can be readily converted into gelatine by simple heating (Zhang et al. 2006).

A stock solution of gelatine (5 mg/ml) was digested with collagenase and afterwards diluted in several steps. The individual dilutions were characterized as described above by positive ion MALDI MS with reference to a known amount of Arg-Gly-Asp (Fig. 5). The trace in Fig. 5a corresponds to 1.67 μg, that in Fig. 5b to 1.34 μg, that in Fig. 5c to 1.0 μg, and that in Fig. 5d to 0.83 μg gelatine (final amount on the MALDI target). Similar mass spectra as in the case of the isolated peptides can obviously be obtained, and concentration-dependent changes are clearly detectable. This is a strong indication that other peptides that are also generated upon the digest do not have a significant influence on Gly-Pro-Hyp determination. We assume that this is attributable to the significantly higher concentration of Gly-Pro-Hyp in comparison with other peptides. We also investigated whether a pronounced influence of additional GAGs exists, because a major contribution of these sulfated carbohydrates is expected if tissue samples are investigated. For this purpose, the samples (the spectra of which are shown in Fig. 5) were re-investigated in the presence of an excess of chondroitin sulfate, the most abundant GAG of cartilage. The presence of chondroitin sulfate results only in negligible spectral changes (data not shown). However, this is not surprising because the analyzed compounds are neutral or even acidic oligopeptides and thus do not bind to the negatively charged chondroitin sulfate.

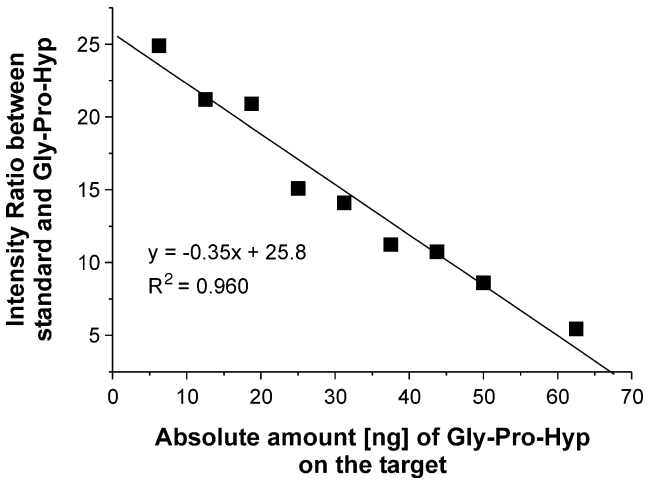

Fig. 4 Quantitative data analysis of the positive ion MALDI-TOF mass spectra as, for example, illustrated in Fig. 3. The ratios between the intensities of the standard tripeptide and Gly-Pro-Hyp are plotted against the absolute amount of Gly-Pro-Hyp on the MALDI target. Note that a clear linear relationship exists between the two parameters and can be described by the given formula. Standard deviations of the individual measurements are ±15%

In the case of gelatine, a linear relationship (Fig. 6) can obviously be also obtained, and the achievable accuracy of the data is similar to the data obtained with the isolated tripeptide. However, significantly higher amounts of gelatine are evidently required in order to obtain analyte/standard ratios comparable with those shown in Fig. 4. Several explanations can be proposed: (1) the used gelatine is a crude product and might contain impurities that may negatively affect the determination of the total amounts; (2) although a high gelatine concentration was used, the actual

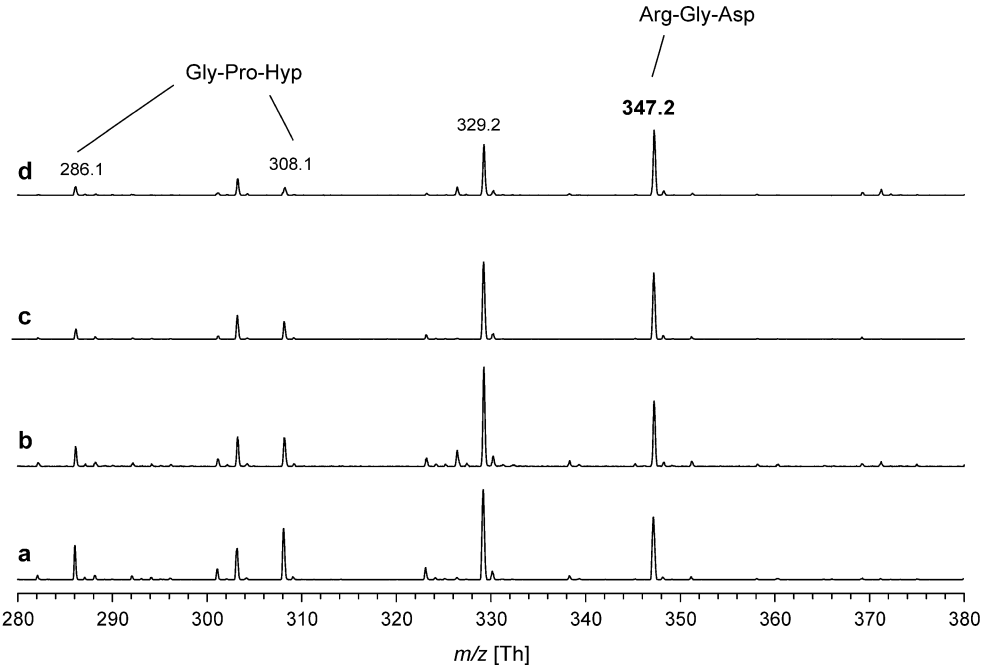

Fig. 5 Selected positive ion MALDI-TOF mass spectra of gelatine digested by bacterial collagenase. Amounts of gelatine (final amount on the MALDI target) of 1.67 μg (**a**), 1.34 μg (**b**), 1 μg (**c**), and 0.83 μg (**d**), i.e., 1 μl of 1.67, 1.34, 1.0, and 0.83 mg/ml solutions, were used, respectively. The stock concentration of the standard was 0.25 mg/ml, and the final concentration 0.083 mg/ml. Subsequently, all samples were mixed 1:1 (v/v) with the matrix solution. The peaks that were used for subsequent detailed analysis are explicitly marked. The peak at m/z=329.2 most probably stems from Gly-Pro-Arg but was not used for further analysis

measured quantities represent only the amount of Gly-Pro-Hyp that is generated by collagenase digestion, whereas all further digestion products have been neglected. Assuming that no cleavages are missed and considering the primary sequence of collagen/gelatine (available from http://www.uniprot.org), one can calculate that only about 1.6% of the gelatine is converted into Gly-Pro-Hyp. This is slightly lower, but still comparable, with the data determined by Schrohenloher et al. (1959). Although a more detailed investigation of the Gly-Pro-Hyp yield was beyond the scope of this study, the small yields of Gly-Pro-Hyp give a reasonable explanation as to why higher amounts of gelatine in comparison with the isolated tripeptide are required in order to obtain the same intensity ratios. Since the water content of the gelatine was also not carefully controlled, absolute quantities should be regarded with caution, and a calibration curve should be recorded with the sample of interest. We will show below that ^{1}H NMR spectroscopy is a convenient method to determine absolute amounts of amino acids, and that these concentrations can be used to correct the amounts of the applied gelatine. NMR has the considerable advantage that diverse amino acids can be simultaneously determined, and an estimation of residual (unwanted) oligopeptides is possible. However, if NMR is not available, calibration can also be performed by the classical determination of the Hyp content subsequent to acidic hydrolysis of the sample.

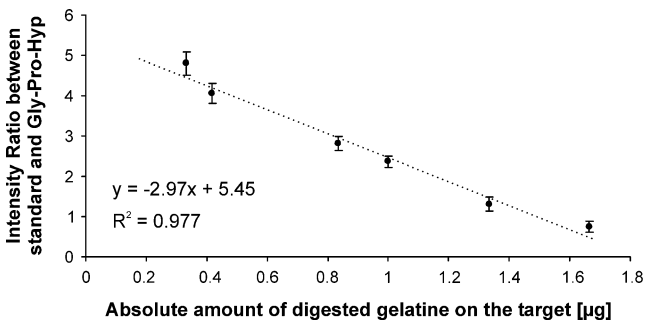

Fig. 6 Quantitative data analysis of the yield of the Gly-Pro-Hyp tripeptide obtained by enzymatic digestion of gelatine. Complete digestion of the gelatine is assumed, and the data analysis was performed as described in Fig. 4. Note that higher amounts of gelatine are required in comparison with the isolated tripeptide, because only a small part of the gelatine is converted into the Gly-Pro-Hyp that is used as a concentration standard. *Error bars* represent the standard deviations of three independent measurements

Finally, the analysis of the collagen content of a selected cartilage sample was also performed. As the exact collagen content of the used porcine articular cartilage sample was not known, this determination was performed initially by means of ^{1}H NMR spectroscopy. In order to obtain the isolated amino acids that could be used as a measure of the collagen moiety (particularly Hyp and Gly), the cartilage tissue was hydrolyzed in advance by treatment with 6 M HCl, and this solution was directly assessed by high resolution NMR.

In Fig. 7, the ^{1}H NMR spectra of an artificial mixture of Gly and Hyp (5 mg/ml each; Fig. 7a), gelatine (Fig. 7c), and porcine articular cartilage (Fig. 7d) are shown. The trace in Fig. 7b corresponds to a simulated spectrum of the Gly/Hyp mixture. The concentrations of Gly and Hyp can evidently be readily determined by comparing the integral

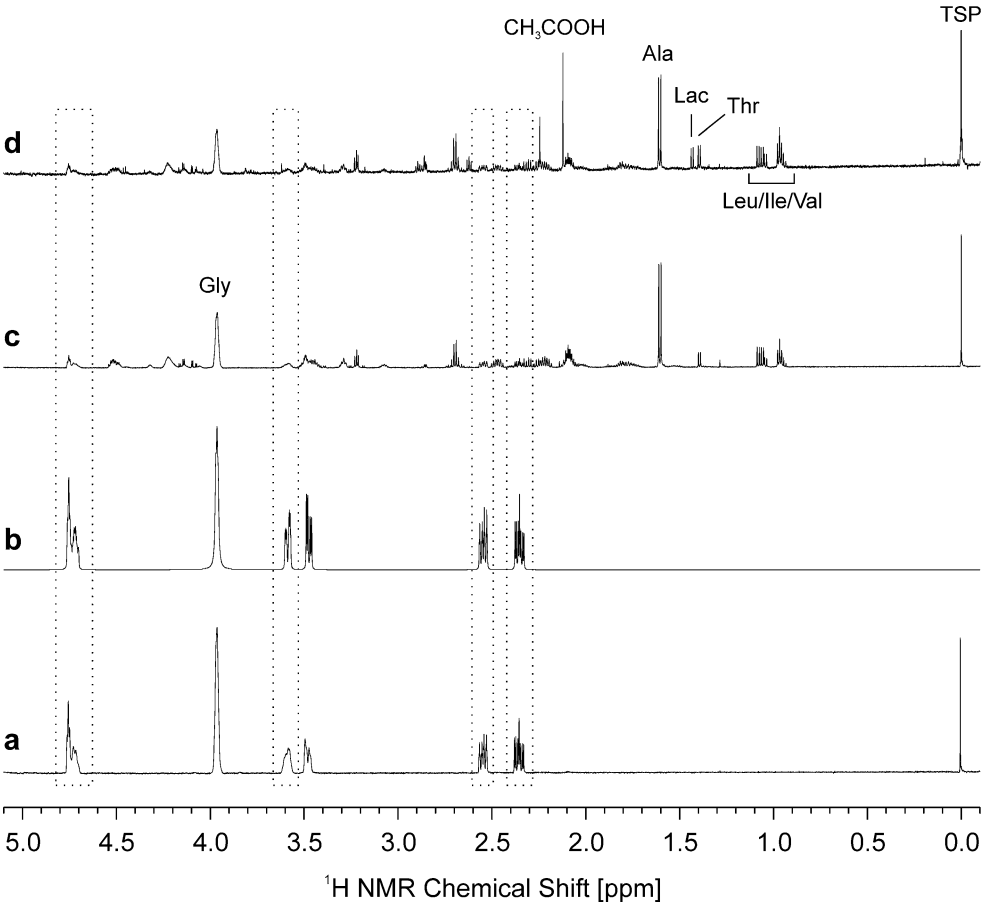

Fig. 7 High resolution ^{1}H NMR (600.13 MHz) spectra of an artificial mixture of glycine and hydroxyproline (**a**), gelatine (**c**), and porcine articular cartilage (**d**). The trace in **b** corresponds to the simulated spectrum of the Gly/Hyp mixture and is in excellent agreement with the real spectrum. All spectra were recorded in the presence of 6 M HCl, which was applied to completely hydrolyze the polymers. The concentrations of gelatine and cartilage were 8.3 mg/ml, and the concentrations of Gly and Hyp were 5 mg/ml each. The volume was 600 µl. The lack of broad resonances clearly indicates that all compounds were broken down to the level of individual amino acids. Gly and Hyp concentrations were determined by comparing their integral intensities with the intensity of the trimethylsilyl group of the TSP standard. The *dotted boxes* indicate the resonances that were used for the determination of the Hyp concentration

intensities of a well-resolved Hyp resonance with the intensity of the trimethylsilyl residue of the TSP standard that provides an intense resonance at 0.00 ppm. The Hyp resonances only slightly overlap with resonances of other amino acids. Notably, the intensity of the TSP resonance is only apparently high, because its intensity corresponds to nine equivalent protons. Therefore, all the intensity is present in a single peak of high intensity. Both, the Gly and the Hyp concentrations can be easily determined from the spectra of the gelatine and the cartilage. The spectra of the gelatine and the cartilage are nearly identical, and this is a clear indication that collagen is the most abundant constituent of cartilage.

However, in the cartilage sample (Fig. 7d), an additional, very narrow resonance is notable at 2.12 ppm; this can be assigned to the acetic acid that is generated by the acidic hydrolysis of the N-acetyl groups of the GAG of cartilage. The absence of this resonance in the gelatine sample clearly establishes the absence of GAG in this sample. From these data, the Hyp content of cartilage was calculated to be 1.44 mmol/l (0.19 mg/ml) and that of gelatine 10.6 mmol/l (1.39 mg/ml). Assuming that the Hyp concentration multiplied by a factor of 7.5 (Edwards and O'Brien 1980) gives the collagen content of the corresponding sample, one can calculate 10.43 mg/ml for the collagen content of the gelatine sample. This is in good agreement with the used sample (10 mg/ml). The cartilage collagen concentration (1.43 mg/ml) is also in acceptable agreement with the cartilage concentration within the sample (10 mg) assuming a water content of 70% by weight and a 70% contribution of collagen to the dry weight of cartilage as approximate values. Of course, there is no need to use such a highly sophisticated method as NMR, but a spectrophotometric method for the determination of the Hyp content would be sufficient in order to calibrate the MALDI data and to account for the water content of the used tissues. Although the Gly content might be similarly and even more accurately determined because of the slight overlap between the Hyp and other resonances, we regard the Hyp content as more reliable, because Gly might also stem from other proteins, whereas Hyp is specific to collagen. Unfortunately, however, NMR is only applicable if sufficiently large amounts of tissues are available.

These data were now used for the estimation of the collagen content of the cartilage sample by MALDI-TOF-MS. For this purpose, the cartilage was digested by collagenase, and the concentrations were determined as described above (Fig. 8). The ratios between the Gly-Pro-Hyp peaks and the intensity of the Arg-Gly-Asp peptide standard were plotted against the amount of the cartilage.

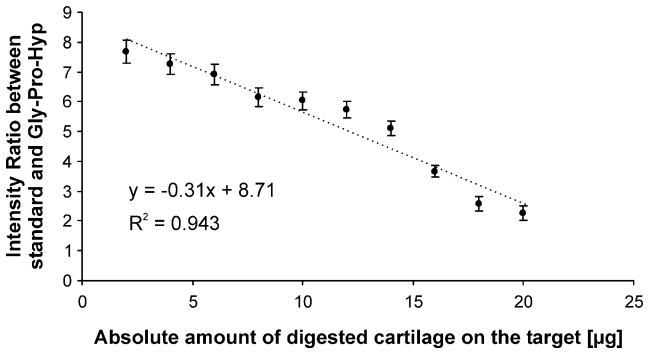

Fig. 8 Quantitative data analysis of the yield of the tripeptide Gly-Pro-Hyp obtained by enzymatic digestion of porcine articular cartilage. Complete digestion of the cartilage is assumed, and the data analysis was performed as described in Fig. 4. Note that the data along the *x-axis* correspond to the wet weight of the cartilage as determined by weighing. *Error bars* represent the standard deviations of three different measurements

From a comparison of the slopes of the linear fits indicated in Figs. 6, 8, the cartilage data must be divided by a factor of 9.7, as the collagen constitutes only a small part of the wet weight of cartilage. As the water content of cartilage might vary significantly depending on the age of the animal, we stongly recommend that an appropriate calibration curve is recorded for each sample of interest. Similar data to that in the case of the gelatine (cf. Fig. 6) are obtained following this division step. Because of the linearity of the obtained graph, the method is obviously suitable for the determination of the collagen contents of tissues that are only available in small amounts.

A direct comparison between the compositional data determined either by MALDI MS or NMR spectroscopy is shown in Fig. 9:

From the data shown in Fig. 9, we can see that MALDI MS and NMR provide similar data. Thus, both methods can be used, although they obviously possess strongly different sensitivities. Additionally, different assumptions have to be made. On using NMR, the Hyp content is determined subsequent to acidic hydrolysis. Thus, the calculation of the collagen content relies on the assumption that Hyp constitutes each 10th amino acid residue. On the other hand, the MALDI-based determination assumes complete digestion by collagenase, and a suitable calibration curve is imperative. Whether all these assumptions are always valid is unclear.

In our opinion, the developed MALDI-MS method is particularly useful if relative changes of the collagen contents of a certain sample are of interest; for instance, the synthesis of collagen by chondrocytes or stem cells that have differentiated into chondrocytes can be determined with an accuracy of ±15% (data not shown). Such data can be easily obtained by using the calibration given in this manuscript. However, if absolute amounts of collagen are required, we strongly suggest that a suitable calibration curve is recorded with a sample of known composition. This is particularly necessary in order to correct for differences in the water content of the samples of interest. According to our experience, the complete removal of water from biomaterials is extraordinarily difficult because a considerable water fraction is tightly bound to the GAG of cartilage.

Concluding remarks and outlook

In this study, we have shown that nanogram amounts of the collagen-derived tripeptide Gly-Pro-Hyp can be determined by MALDI-TOF-MS analysis of tissue digests. However, as only about 1.6% of the total collagen is converted into Gly-Pro-Hyp, this indicates that microgram quantities of the collagen are necessary. This limitation is at least partially caused by the reduced sensitivity of the TOF mass analyzer in the small mass range. Nevertheless, we have accepted this disadvantage because MALDI-TOF-MS devices are commonly available and widely used.

Even larger amounts of cartilage are necessary because of its water and GAG content. A commercially available, inexpensive tripeptide has been used here as an internal standard of known concentration. This internal standard seems the most straightforward approach to compensate for the various ion concentrations within the sample and potential

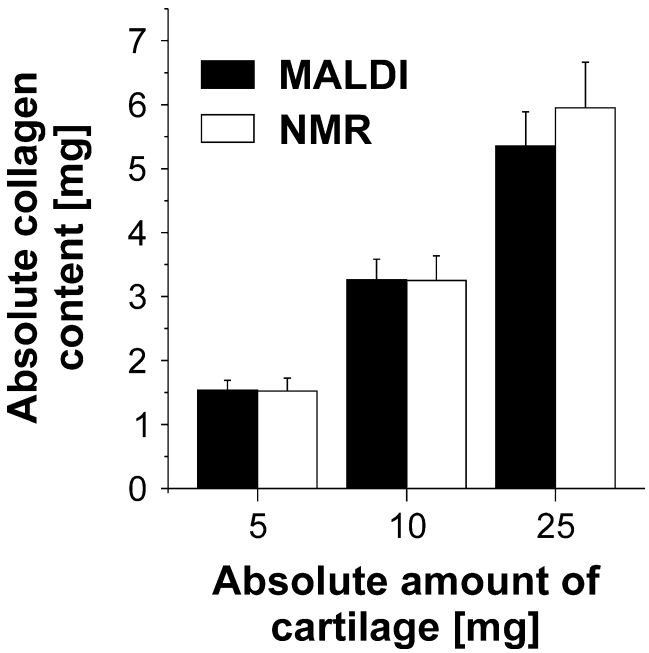

Fig. 9 Comparison of MALDI and NMR determinations of absolute collagen contents in porcine articular cartilage. The absolute amounts of cartilage that are plotted along the *x-axis* were determined by weighing. Collagen contents were determined subsequent to acidic hydrolysis (NMR) or collagenase digestion (MALDI)

changes in the laser fluence. The same method is suitable to determine microgram amounts of gelatine and cartilage collagen after collagenase digestion. The applicability to gelatine and cartilage is a clear indication that this new method is robust and not affected to a large extent by the actual composition of the tissue sample. In particular, the GAG content of cartilage does not affect the concentration determination of the collagen to a major extent. Therefore, this method is basically applicable to native tissue samples and to bioengineered tissues with widely variable collagen/GAG ratios. Preliminary data obtained from ECMs generated by stem cells that have differentiated into chondrocytes are promising and will be reported in due course.

The suggested assay is fast, convenient, and inexpensive and is thus a suitable alternative to the classical Hyp determination that is, in our opinion, more time-consuming and tedious. The most important prerequisite is a sufficient excess of collagenase for the complete digestion of the collagen/gelatine or of the tissue sample of interest. The absence of larger collagen fragments can be easily verified by SDS-PAGE, by NMR spectroscopy, or by the investigation of the higher *m/z* ranges of the MALDI mass spectra.

Nevertheless, we must explicitly state that the differentiation of the individual collagen types cannot be performed by this simple approach because of the low specificity of the bacterial collagenase that affects all collagen subtypes to a comparable extent. This is a particular disadvantage with regard to the analysis of bioengineered cartilage for two reasons. First, different collagen types possess different mechanical properties and, thus, detailed compositional information about the sample of interest is of importance. Second, stem cells or chondrocytes (the only cartilage cell) are often seeded in a collagen gel (Koga et al. 2008; Schulz and Bader 2007) because the presence of ECM components enhances the extent of ECM generation. Therefore, it is impossible to differentiate the collagen in the artificial collagen gel from the *de novo* synthesized collagen by using the above-described assay, and only an overall estimation of the collagen content is possible.

For the differentiation of the individual collagen types, more selective enzymes or possibly chemical digestion methods are required. We are currently evaluating an assay that enables the differentiation of the different collagen types.

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