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^{1}H and ^{13}C HR-MAS NMR investigations on native and enzymatically digested bovine nasal cartilage

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Abstract

Rheumatic diseases are accompanied by a progressive destruction of the cartilage layer of the joints. Despite the frequency of the disease, degradation mechanisms are not yet understood and methods for early diagnosis are not available. Although some information on pathogenesis could be obtained from the analysis of degradation products of cartilage supernatants, the most direct information on degradation processes would come from the native cartilage as such. We have used ^{1}H as well as ^{13}C HR-MAS (high resolution magic angle spinning) NMR spectroscopy to obtain suitable line-widths of NMR resonances of native cartilage. 1D and 2D NMR spectra of native cartilage were compared with those of enzymatically-treated (collagenase and papain) samples. In the ^{1}H NMR spectra of native cartilage, resonances of polysaccharides, lipids and a few amino acids of collagen were detectable, whereas the ^{13}C NMR spectra primarily indicated the presence of chondroitin sulfate. Treatment with papain resulted only in small changes in the ^{1}H NMR spectrum, whereas a clear diminution of all resonances was detectable in the ^{13}C NMR spectra. On the other hand, treatment with collagenase caused the formation of peptides with an amino acid composition typical for collagen (glycine, proline, hydroxyproline and lysine). It is concluded that the HR-MAS NMR spectra of cartilage may be of significance for the investigation of cartilage degradation since they allow the fast evaluation of cartilage composition and only very small amounts of sample are required.

Keywords: Cartilage; NMR spectroscopy; Papain; Collagenase; HR-MAS NMR

1. Introduction

Rheumatic diseases are a major cause of disability and early retirement in the industrialized countries and are, thus, of great socioeconomic significance [1]. The progressive diminution of the thickness of the cartilage layer is a typical marker of this disease, whereas details of the mechanisms of cartilage degradation remain yet widely unknown [1].

The biochemical analyses of pathologically modified synovial fluids from patients suffering from rheumatic diseases were very helpful to gain further insights into the mechanisms of cartilage degradation since fragmentation products of cartilage are accumulated in the synovial fluid [2,3]. These investigations indicated that products derived from neutrophilic granulocytes play a prominent role for cartilage degradation [4].

The contribution of reactive oxygen species like hypochlorous acid (HOCl) [5] or hydroxyl radicals ($HO^{\cdot}$) [6] as well as different enzymes [7,8] to the degradation of isolated polysaccharides, cartilage specimens and synovial fluids was also intensively studied by high resolution NMR spectroscopy to clarify which signals may be used as 'markers' for cartilage degradation [2–8]. Sensitivity of NMR is enhanced by the presence of relatively mobile and, therefore, intense N-acetyl groups of cartilage polysaccharides [2–4]: When depolymerization of cartilage polysaccharides occurs, the comparably sharp resonances of degradation products are easily detectable in comparison to the broad resonances of native cartilage polysaccharides. These investigations were quite useful for a molecular understanding of arthritis [1].

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However, these experiments are limited in the sense that they were exclusively performed on the supernatants of cartilage samples after the treatment with different agents. Therefore, the obtained results are strongly influenced by further parameters, especially the extraction behaviour of cartilage tissue. On the other hand, the investigation of the native cartilage samples would provide a more direct approach to the in vivo situations relevant for cartilage degradation. Unfortunately, standard high-resolution ^{1}H NMR spectroscopic analysis of cartilage samples is not possible since only broad, less informative resonances can be obtained due to significant chemical shift anisotropy and residual dipolar coupling contributions for motionally constrained species [9]. Therefore, ^{13}C NMR instead of ^{1}H has been mainly used in the past [10,11].

One modern approach to overcome problems with line-width of ^{1}H NMR resonances is the application of the high resolution magic-angle-spinning (HR-MAS) technique. Here, in contrast to standard solid state NMR no strong decoupling is used. The solid-like sample is, however, spun at high speeds under the magic angle of 54.7° with respect to the applied magnetic field. This minimizes the line-broadening effects of chemical shift anisotropy and dipolar coupling considerably and, therefore, sharper NMR resonances can be obtained [9]. One further advantage of this method is the small size of the used MAS rotors and the resulting very small amounts of sample that are required.

To our knowledge, however, cartilage samples have not yet been investigated by the HR-MAS technique. Therefore, the aim of the present paper is the evaluation of cartilage by HR-MAS NMR spectroscopy. Additionally, enzymatic digestions of cartilage specimens with proteolytic enzymes are also performed and the cartilage samples were analyzed by HR-MAS ^{1}H and ^{13}C NMR spectroscopy. Since the collagen moiety of cartilage is not detectable without the application of high-power decoupling [12] as well as for the optimization of methodology, bovine nasal cartilage was investigated. This cartilage contains higher amounts of polysaccharides [13] and less collagen than the physiologically more relevant articular cartilage [14] but is in principle comparable.

2. Material and methods

2.1. Chemicals

Chemicals for buffer preparation (NaCl, Na_2HPO_4 and KH_2PO_4) and for NMR spectroscopy (deuterated water with an isotopic purity of 99.95% and 3-trimethylsilyl-propionate (TSP)), as well as chondroitin sulfate from bovine trachea and gelatine from calf skin of the highest commercially available purity were obtained from Fluka Feinchemikalien GmbH, Neu-Ulm, Germany. They were used without any further purification.

The enzymes (collagenase from *Clostridium Histolytikum* and papain from *Cacarica Papaya*) were purchased from Fluka and also used without further purification. Solutions were prepared containing one enzymatic unit (1 U) per milliliter. These enzyme solutions were prepared immediately prior to use in 50 mM phosphate buffer containing 154 mM NaCl. The buffer was prepared in D_2O to minimize the water content of the sample.

2.2. Cartilage preparation

Fresh bovine nasal cartilage was obtained from healthy juvenile animals ($\sim$ 12 months old) within a few hours after slaughter. After the surrounding soft tissue was removed, the cartilage was cut into small pieces (cubes of $\sim$ 1.5 mm diameter), which were used immediately for all further experiments (however, freezing the cartilage over a few weeks did not markedly change its NMR properties).

2.3. Incubation conditions

100 mg (wet weight) cartilage specimens and 800 µl buffer (50 mM phosphate, 154 mM NaCl, pH 7.4) containing the corresponding enzymes were mixed in small plastic tubes and incubated at 37°C in a water bath for 4 h. Control incubations were performed with buffer (without enzyme). Although the activities of collagenase and papain are known to be Ca^{2+}- [15] or thiol-dependent [16], the addition of $CaCl_2$ or cysteine did not markedly enhance the enzymatic effects. We assume that a sufficient amount of Ca^{2+} ions and thiols is released from the cartilage samples. Therefore, no external additions are required [8].

After incubation, samples were spun down to separate the supernatant from the insoluble tissue. The resulting clear, slightly yellow supernatant was analyzed for means of comparison directly by NMR and these spectra agreed very well with previously published data [7,8]. The cartilage specimens were washed three times with deuterated water to remove traces of enzymes and were subsequently filled into a 4 mm MAS rotor for HR-MAS NMR.

2.4. NMR measurements

All NMR measurements were conducted on a Bruker DRX-600 spectrometer operating at 600.13 MHz for ^{1}H. All spectra were recorded at 37°C (310 K) using a broadband NMR probe. Typically, 0.54 ml supernatant from the incubation experiments and 60 µl of a 20 mM 3-trimethylsilyl-propionate (TSP) solution were placed

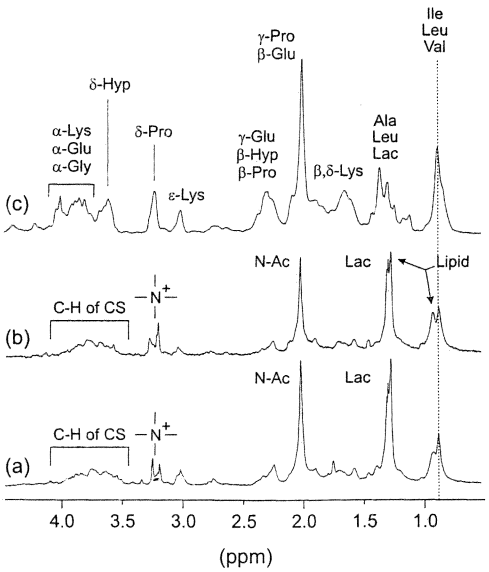

Fig. 1. HR-MAS ^{1}H-NMR spectra of bovine nasal cartilage after incubation in 0.8 ml 50 mM phosphate buffer containing 154 mM NaCl at 37°C for 4 h (a). (b–c) were recorded in the presence of 1 U papain and 1 U collagenase, respectively. Abbreviations used in peak assignment: CS, chondroitin sulfate; N-Ac, N-Acetyl; Lac, Lactate; Hyp, Hydroxyproline; N^+, quaternary ammonia groups. All amino acids are labelled according to the three letter code.

in a 5 mm NMR tube. The residual water signal (HDO) was suppressed by presaturation on the water resonance frequency. 128 transients were acquired for each spectrum 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. On selected samples, two-dimensional ^{1}H-^{1}H correlation spectroscopy (COSY) was performed [17]. Here, FIDs were acquired with 32 scans and a spectral width of 3000 Hz into 2 K data points for 256 values of the evolution time. A sine-bell transformation was used in both dimensions prior to Fourier transformation. Partially relaxed ^{13}C-NMR spectra were obtained at 150.92 MHz. Data were recorded with a spectral width of 200 ppm, 32 K time-domain data points with a pulse angle of 45° (3.3 μs pulse) and a repetition time of 2 s. Usually 1–8 K transients were accumulated with low-power broadband ^{1}H decoupling. All free induction decays were processed with a 5 Hz line-broadening. Chemical shifts were referenced to internal sodium 3-trimethylsilyl-propionate (TSP) at a final concentration of 2 mM.

^{1}H and ^{13}C HR-MAS NMR spectra of cartilage were acquired with a 4 mm dual (^{13}C-^{1}H) MAS probe. The spin rate was 5000 Hz and the spectra were acquired at a temperature of 310 K. All experimental parameters were the same as described above for high-resolution NMR. Since no additional standard was available under HR-MAS NMR conditions, the methyl protons of lactate ($\delta = 1.31$ ppm) served as internal standard [18]. Since it is known that the N-acetyl methyl group of CS is only slightly influenced by changes of the chemical environment, this resonance was used for the calibration of the ^{13}C NMR spectra [19].

2.5. MALDI-TOF mass spectrometry

To check the contribution of lipids to NMR spectra, lipids of cartilage were extracted according to Bligh & Dyer [20] by a 1:1:0.9 (v:v:v) mixture of methanol, chloroform and water. After separation of both layers, the chloroform phase was immediately used for mass spectrometry. All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA). Cartilage extracts were directly applied on the sample plate as 1 μl droplets, followed by the addition of 1 μl matrix solution. For all samples a 0.5 mol/l 2,5-dihydroxybenzoic acid (DHB) solution in methanol containing 0.1% trifluoroacetic acid was used as matrix solution. Subsequently, samples were rapidly dried under a moderate, warm stream of air. Details of mass spectrometric investigations are given elsewhere [21,22].

3. Results

3.1. Proton HR-MAS spectroscopy of cartilage

Although cartilage has already been characterized by different NMR methods, it has not yet been investigated by high-field, high-resolution proton NMR spectroscopy. Most probably this has not yet been done since proton NMR spectra are strongly influenced by sample inhomogeneities. Therefore, it was our first aim to investigate if the application of MAS conditions may help to improve the quality of NMR spectra.

The HR-MAS proton NMR spectra of bovine nasal cartilage subsequent to: (a) buffer treatment; or (b) digestion with papain, respectively; (c) collagenase are shown in Fig. 1. These enzymes were chosen because of their known selectivities towards the proteins of cartilage [7,8]. Clear differences between cartilage specimens subsequent to enzymatic digestion could be monitored. In all cases, however, spectra were dominated by three peaks centred at ~2.0, 1.3 and 0.9 ppm. In the case of the buffer- and papain-treated cartilage also the lactate resonance at 1.31 ppm is clearly detectable as a doublet [18]. This confirms the considerable improvement of spectral quality by the application of HR-MAS conditions since at least coupling constants of ~7 Hz can be clearly detected. This would not be possible if the cartilage spectra would be recorded without HR-MAS (data not shown).

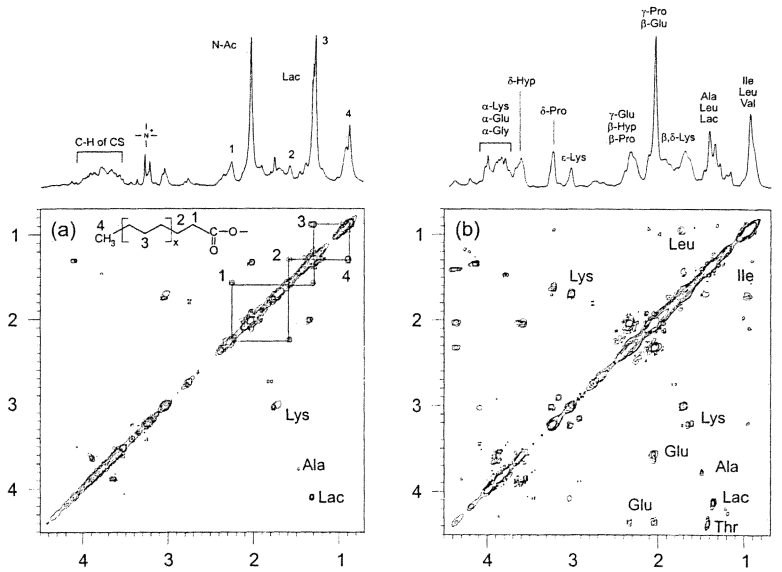

Fig. 2. ^{1}H-^{1}H COSY NMR spectra of bovine nasal cartilage after incubation with buffer (a) and collagenase (b). Only the most prominent compounds are assigned in the figure. All amino acids are labelled according to the three letter code. The squares in (a) indicate the connections between different protons, which were used to assign these resonances to lipids.

The intense resonance at ~2.0 ppm was assigned to the N-acetyl side groups of the polysaccharides of cartilage. These possess a tremendous mobility and are, therefore, most easily detectable due to their long T_2 relaxation times [2–4]. This is the reason why this resonance is easier detectable than the residual -C-H groups of the polysaccharides of cartilage, which show only broad resonances between ~3.5 and 4.0 ppm. It will be outlined below in more detail that upon enzymatic digestion also amino acids of collagen may contribute to this resonance. Additionally, there are besides some further resonances of lower intensity two intense resonances at ~1.3 and 0.9 ppm, which change clearly upon enzymatic digestion. Since especially the treatment with collagenase induces major differences (especially the enhancement of the resonance at 0.9 ppm) it is most likely that this peak contributes to protons of the collagen of cartilage. On the other hand, the chemical shifts of both resonances are in agreement with the methylene and methyl groups of fatty acids of lipids.

To investigate the origin of both resonances in more detail, we have also recorded the corresponding two-dimensional proton NMR spectra. In Fig. 2 the COSY NMR spectra of cartilage incubated with pure buffer (a) and after collagenase treatment (b) are shown. Projections on the X-axis are the same spectra as shown in Fig. 1. It is clearly evident that the number of detectable cross-peaks is drastically enhanced when the cartilage sample is digested with collagenase, resulting in the cleavage of the collagen backbone under the formation of smaller peptides. In contrast, the COSY spectrum of cartilage incubated solely with buffer shows only a few cross-peaks, that are mainly due to the presence of lactate (4.09 and 1.31 ppm) and alanine (3.78 and 1.48 ppm). The third resonance that shows cross-peaks belongs to the ε protons of lysine. Since these protons are far away from the peptide backbone, they possess high mobility and are, therefore, most easily detectable.

Since the majority of the other resonances represents singlets, the corresponding compounds cannot yet be completely assigned. However, it is obvious that a scalar coupling exists between the peaks at 2.24 and 1.60 ppm as well as the peaks at 1.29 and 0.90 ppm (cf. squares and assignment in Fig. 2(a)). This is a very typical coupling pattern of lipids and, therefore, the corresponding peaks must be explained by the presence of lipids [23]. Accordingly, the sharp singlets at 3.25 and 3.19 ppm detectable in Fig. 1(a–b) would represent the ammonia head groups of, e.g. phosphatidylcholines, which occur in high amounts in the cell membranes. The presence of phospholipids will be additionally confirmed by the application of mass spectrometry.

In comparison to the buffer-treated cartilage, the collagenase-digested cartilage (Fig. 2(b)) shows by far more cross peaks which can be mainly assigned to different amino acids of fragmentation products of collagen. This assignment is in complete agreement with previous NMR studies on characteristic peptides of collagen containing mainly lysine, glutamic acid, glycine, proline and hydroxyproline [24]. These amino acids are labelled in Fig. 2(b) according to the three letter code.

Although the presence of those amino acids is not surprising since they are very abundant in collagen, there are also the typical coupling patterns of leucine,

valine, isoleucine and alanine detectable. Although these amino acids are not very abundant in collagen, their less polar residues clearly contribute to the comparably intense peaks at 1.3 and 0.9 ppm. This indicates that the collagenase digestion requires changes towards peak assignment: Whereas those resonances represented lipids in buffer-treated cartilage, they correspond to amino acid residues of collagenase-digested cartilage. Also the resonance at ~2.0 ppm must now be attributed to the protons of different amino acids, whereas polysaccharides do only play a minor role in that case.

Unfortunately, even by means of two-dimensional NMR spectroscopy, spectra in the range between ~3.5 and 4.0 ppm are difficult to interpret, since in that spectral region an overlap of the α-CH groups of amino acids (respectively peptides) and the CH groups of carbohydrates of cartilage occurs. The amount of detectable carbohydrates is, however, very low under our experimental conditions [8]. We will show below that ^{13}C NMR spectroscopy is much more suitable for that purpose and that under these conditions an assignment of the majority of these resonances is possible.

3.2. Mass spectrometry of organic cartilage extracts

To support our assumption that resonances of cartilage are caused to some extent by the presence of lipids, organic extracts of cartilage were additionally investigated by mass spectrometry.

Fig. 3 shows the positive ion MALDI-TOF mass spectrum of bovine nasal cartilage, whereas the negative ion mass spectrum (recorded under identical conditions) did not show any peaks.

Obviously, a large number of different peaks, representing marked differences in fatty acid composition of

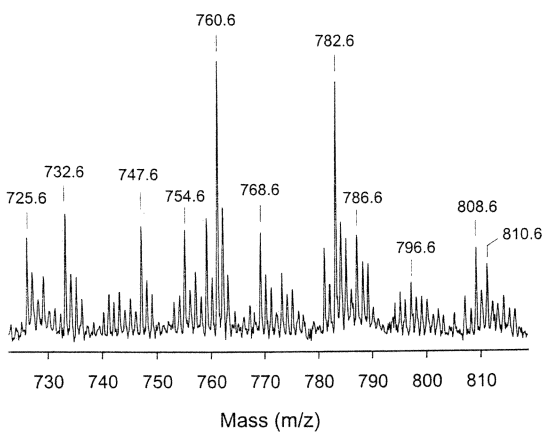

Fig. 3. Positive ion MALDI-TOF mass spectrum of the organic extract (obtained by the Bligh & Dyer method) of native bovine nasal cartilage. The spectrum was recorded in a matrix of 0.5 M 2,5-dihydroxybenzoic acid in methanol. All peaks are labelled according to their m/z ratio.

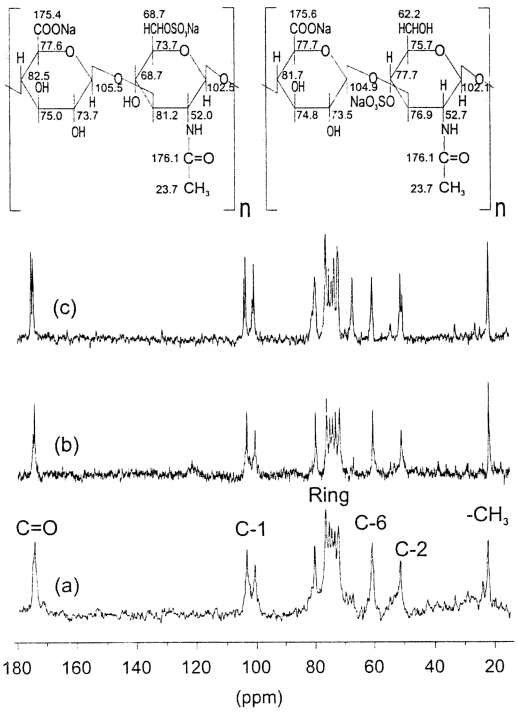

Fig. 4. ^{13}C NMR spectra of bovine nasal cartilage under standard high-resolution (a) and HR-MAS conditions (b) as well as of chondroitin sulfate (8 wt.% in D_2O; (c)). In (a), cartilage was allowed to free swelling in D_2O. 1 K accumulations were sufficient for (a) and (c), whereas the lower sample volume in (b) required 8 K accumulations. The chemical structure of both chondroitin sulfate isomers as well as the assignment of all ^{13}C NMR resonances is given at the top of the figure.

phospholipids is present in the cartilage extracts. Most of those peaks, however, are caused by the presence of phosphatidylcholine and only a few peaks contribute to sphingomyeline. The following major peaks could be identified: Sphingomyeline (SM) yields only two peaks, one at 725.6 and a second one at 747.6. All further peaks are due to different phosphatidylcholines (PC) and assigned as follows: 732.6 (14:0/18:1), 760.6 (16:0/18:1), 782.6 (16:0/20:4), 786.6 (18:0, 18:2) and 810.6 (18:0/20:4). Therefore, this spectrum is in good agreement with the previously described spectrum of PC from egg yolk [21]. The exclusive presence of SM and PC may be easily explained by the fact that MALDI-TOF mass spectrometry detects phospholipids containing one preformed positive charge (like in PC and SM) more easier than other phospholipids [25,26]. Therefore, the assignment of NMR resonances to lipids is also in agreement with mass spectrometric data.

3.3. ^{13}C NMR spectroscopy of cartilage

Since carbon NMR spectra are less influenced by inhomogeneities of the sample, these spectra do not have to be absolutely recorded under MAS conditions. However, also ^{13}C NMR spectra are improved under

MAS conditions. Fig. 4 shows the comparison between ^{13}C NMR spectra of bovine nasal cartilage obtained under different experimental conditions. Spectrum (a) was recorded using a standard high-resolution 5 mm NMR probe, whereas (b) represents the same sample under HR-MAS conditions (5000 Hz spinning frequency). For means of comparison in (c) the ^{13}C NMR spectrum of an aqueous chondroitin sulfate solution (8% w/w) is also given. This concentration was used since it is comparable to the concentration of chondroitin sulfate in cartilage. The chemical structure of both chondroitin sulfate isomers (4- and 6-sulfate) as well as the chemical shifts of all ^{13}C resonances are shown at the top of the figure. The assignment of all resonances was performed according to previously published data [11,19,27,28]. Most likely due to the higher field strength of our spectrometer, spectra are by far higher resolved in comparison to [10].

Although spectra resemble each other closely, one should notice upon the comparison of cartilage spectra (a) and (b) on the one hand and the chondroitin sulfate spectrum (c) on the other hand that some resonances are lacking in the cartilage spectra. This especially concerns the resonance of the C-6 of the N-acetylgalactosamine in chondroitin 6-sulfate ($\delta = 68.7$ ppm) and confirms that bovine nasal cartilage contains only low amounts of chondroitin 6-sulfate. This chondroitin sulfate isomer as well as keratan sulfate and hyaluronan are not detectable in the cartilage spectra that therefore represent mainly chondroitin 4-sulfate.

Although spectra were not obtained under complete relaxation conditions (since the CO and the CH_3 group possess T_1 relaxation times of ~ 2 s [11], a pulse delay of only 2 s is not sufficient to fulfil this criterion) a qualitative analysis of the spectra can be performed. Upon the comparison of spectra (a) and (b) it is obvious that HR-MAS conditions (b) lead to a marked diminution of the line-width of all resonances. Therefore, HR-MAS conditions were used to monitor the effects of enzymatic digestions. However, signal to noise ratio is better in spectrum (a). This is caused by the different amounts of sample, which may be used in both techniques: Since the volume of the MAS rotors is low in comparison to a standard 5 mm NMR sample tube, the achievable signal to noise ratio is poor under HR-MAS conditions.

In Fig. 5, the HR-MAS ^{13}C NMR spectra of bovine nasal cartilage samples after buffer (a), papain (b) and collagenase (c) treatment are shown. Whereas the spectrum of the buffer treated cartilage (a) primarily shows the resonances of chondroitin 4-sulfate, which can be easily assigned by comparison with the corresponding polysaccharide solution (4c), only very weak signals are detectable after treatment with papain (Fig. 5(b)). This is in agreement with the proton HR-MAS spectra, where the papain digestion resulted only in small changes in comparison to the pure buffer-treated cartilage. It is therefore most likely that the papain, because of its ability to cleave the core and the link protein of the proteoglycan aggregate of cartilage [1,8], produces high amounts of soluble chondroitin sulfate (which is, however, still bound to a small protein residue since the papain is not able to cleave any glycosidic linkages). Obviously, the resulting fragmentation products are small enough to diffuse out of the cartilage network, and are, thus, detectable in the corresponding cartilage supernatants, as previously shown [8]. Therefore, the action of papain can be easily explained by the removal of carbohydrates from the cartilage. A completely different situation is, however, found when the cartilage is incubated with collagenase (Fig. 5(c)). In this case, a vast number of intense resonances, that do not correspond to polysaccharides, indicates the formation of different oligopeptides containing high amounts of amino acids like glycine ($\delta = 42.6$ ppm), proline ($\delta =$ 25.0, 30.0, 47.4, 62.3 ppm), hydroxyproline ($\delta = 41.8$, 57.3, 64.2, 74.4 ppm) or alanine ($\delta = 51.5$ and 17.4 ppm), which are known to be abundant amino acids of the collagen of cartilage [8,29]. Therefore, one must also conclude that the extraction behaviour of cartilage is completely different for carbohydrates and proteins: Whereas carbohydrates are easily removed from the cartilage tissue (most probably due to their negative charges), peptides are retained in the cartilage matrix. These results are in agreement with Jelicks et al. [10]. These authors also found in cartilage slices an enhanced protein content subsequent to proteolytic treatment.

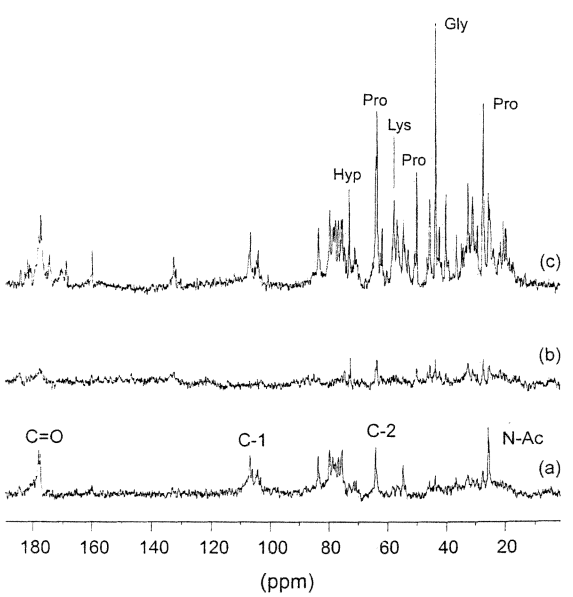

Fig. 5. HR-MAS ^{13}C NMR spectra of bovine nasal cartilage after incubation in 0.8 ml 50 mM phosphate buffer containing 154 mM NaCl at 37°C for 4 h (a). (b–c) were recorded in the presence of 1 U papain or 1 U collagenase, respectively. Spectra represent the average of 8 K accumulations. All amino acids are labelled according to the three letter code.

4. Discussion

Despite the increasing number of patients suffering from rheumatic diseases, knowledge on the mechanism of cartilage degradation as well as diagnostic tools for the assessment of cartilage alterations on a molecular level are still lacking [1]. Most investigations use the analysis of cartilage supernatants to gain knowledge on cartilage degradation mechanisms [5–8]. However, this is a rather indirect approach since changes occurring at the cartilage as such cannot be visualized and results are strongly influenced by the extraction efficiency of the tissue.

Towards NMR analysis, one important progress was the development of the HR-MAS technique that has already been successfully applied to a great number of different tissues [9]. This technique can be used on most NMR devices since it does not apply (as solid state NMR) high-power decoupling but does only rotate the sample in the magic angle. To our knowledge, native cartilage as well as enzymatically-treated cartilage has not yet been characterized by this technique. We have used in this investigation bovine nasal cartilage since it is available in higher amounts than articular cartilage and shows a lower inhomogeneity. The use of bovine nasal cartilage is an established approach in athrosis research [29,30] since only the ratio between glycosaminoglycans (mainly chondroitin 4-sulfate) and collagen (mainly type II) [11,13] is slightly altered in that tissue in comparison to articular cartilage.

Previous ^{13}C NMR studies have indicated that primary resonances of polysaccharides are detectable in native cartilage [10,11]. This is caused by the low concentration of cells and their metabolic products as well as the low molecular mobility of collagen and the resulting broad NMR resonances [11]. Therefore, the detectability of the comparably narrow polysaccharide resonances is highly favoured.

The question, whether the higher sensitivity of proton NMR provides further important information has not yet been addressed. This is most probably due to the fact that proton NMR spectra are by far more influenced by inhomogeneities of the sample and, therefore, HR-MAS conditions are required to obtain reasonable spectra [9]. Highly-resolved ^{1}H NMR spectra of native cartilage can be obtained under HR-MAS conditions and a differentiation between metabolic products of chondrocytes (e.g. lactate) and the polysaccharides of cartilage is possible. Due to its low mobility [12], however, collagen of cartilage yields only one broad resonance at about 0.85 ppm according to the methyl groups of different amino acids [8]. However, phospholipids of chondrocytes are detectable without any problems. This assignment has additionally been confirmed by mass spectrometry of organic cartilage extracts: Although originally developed for protein and peptide analysis, MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight) mass spectrometry is most suitable for the investigation of lipids and especially phospholipids [21,22], since this technique provides both, high mass resolution as well as high sensitivity. From mass spectra it became evident that mainly phosphatidylcholine and sphingomyeline contribute to the lipids of cartilage cells. This is in good agreement with the known lipid composition of cartilage [31].

2D NMR techniques were extremely helpful to assign the resonances of a variety of amino acids after papain and collagenase digestion of cartilage. The effect of papain digestion was very weak and only slight differences in comparison to the native cartilage were detectable in contrast, it has been shown that under these conditions the supernatants of cartilage exhibit intense resonances of polysaccharides [8]. Although papain as well as collagenase possess proteolytic activity, papain mainly cleaves the core and the link protein of cartilage aggregates, whereas it is a unique ability of collagenase to digest also the native collagen of cartilage [1]. Therefore, papain leads to a more expressed formation of low-mass carbohydrates with easily detectable N-acetyl groups (compare the resonance at ~ 2.0 ppm in Fig. 1), whereas collagenase treatment of cartilage results in the formation of oligopeptides. Most amino acids found in the 2D NMR of collagenase-digested cartilage are in agreement with the known amino acid composition of collagen [32]. Although amino acids like leucine, isoleucine or valine are rather scarce in collagen, the methyl groups of these amino acids are clearly detectable in the spectra. Those residues are far away from the peptide backbone, possess higher mobility and give, therefore, highly resolved resonances.

Although ^{13}C NMR spectroscopy is by far less sensitive than proton NMR, the ^{13}C nucleus is more suitable for the detection of polymers (especially in a mixture) since it possesses a broader range of chemical shift and exhibits a minor line-width than the proton (the gyromagnetic ratio of carbon is only one quarter of ^{1}H) [17]. Therefore, ^{13}C NMR spectra of cartilage do not have to be absolutely recorded under MAS conditions to detect the corresponding resonances, although under HR-MAS conditions spectra quality is considerably improved. In the ^{13}C NMR spectra of collagenase digested cartilage, all expected amino acids were found and their intensities were much higher than the resonances of the polysaccharides of cartilage. This clearly indicates that only a smaller moiety of cartilage fragmentation products can be extracted by the solvent. Therefore, analyzing exclusively the supernatant may lead to a considerable underestimation of cartilage degradation. However, this is much more valid for proteins than for glycosaminoglycans. The decrease of chondroitin sulfate resonances after papain digestion indicates that the

majority of carbohydrates is released into the surrounding solution.

Therefore, we conclude that the performed HR-MAS experiments on cartilage are most suitable for the differentiation between the effects of different enzymes. It is our next aim to extend those experiments by high-power decoupling MAS. It shall be tested, if under these experimental conditions, the native collagen of cartilage can be detected by NMR spectroscopy already prior to enzymatic digestion. It is also one of our additional aims to simulate directly the degradation processes occurring during rheumatic diseases by using articular cartilage as well as stimulated neutrophils. Recent results indicate that this might be a very useful approach [7].

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