Nuclear Magnetic Resonance and Mass Spectrometric Studies on the Action of Proteases on Pig Articular Cartilage

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Rheumatic diseases are accompanied by a progradient diminution of the cartilage layer. Unfortunately, degradation mechanisms (role of different enzymes and reactive oxygen species) are not yet understood. Since nuclear magnetic resonance (NMR) spectroscopy was often used for the investigation of synovial fluids, the aim of the present work was to detect cartilage degradation products upon proteolytic digest of cartilage.

Cartilage samples were incubated at 37 °C with phosphate buffer in the absence or presence of different proteases (collagenase, trypsin and papain). Supernatants were subsequently assayed towards their content of carbohydrate and protein degradation products by NMR (^{1}H- and ^{13}C-) and MALDI-TOF mass spectrometry.

Intense resonances of relatively mobile N-acetyl side chains (ca. 2.01 ppm) of polysaccharides of cartilage were only detectable on digestion with papain. The appearance of these resonances indicates intense degradation of the core protein of proteoglycan aggregate of cartilage, whereby polysaccharides are released. Additionally, broad resonances at 0.85 ppm arising from collagen degradation products were observed upon the action of all applied proteases. However, glycine as a marker of exhaustive collagen degradation was only observed, if cartilage was digested by collagenase. Using more vigorous incubation conditions, additionally high-abundant amino acids of collagen (proline, hydroxyproline) could be detected in the ^{13}C-NMR- and the MALDI spectra. The observed differences are correlated with the different selectivities of the applied enzymes.

It is concluded that NMR allows the detection of characteristic protein and polysaccharide degradation products. The observed differences may be of some relevance for the diagnosis of rheumatic diseases.

Introduction

Joint diseases like rheumatoid arthritis are a major cause of disability and early retirement in industrialized countries and are, thus, of great socioeconomic significance. Neutrophils (Brown, 1988) are assumed to play an important role in cartilage degradation by the generation of reactive oxygen species (Allen, 1987; Edwards and Hallett, 1997) and the release of different enzymes (e.g. collagenase and β-galactosidase (Edwards and Hallett, 1997). Although the contribution of such agents is well established in rheumatic diseases (Liszt *et al.*, 1991) the detailed mechanisms of cartilage damage, regulation and defense reactions remain unknown (Lohmander *et al.*, 1992). Cartilage consists (besides 70–80% water) of about 20% collagens and 6% proteoglycans, containing high amounts of negatively charged polysaccharides like chondroitin sulfate and hyaluronic acid (Torchia *et al.*, 1977). Cartilage cells, the chondrocytes, are embedded in this extracellular matrix (see Fig. 1).

The contribution of reactive oxygen species like hypochlorous acid (HOCl) or hydroxyl radicals (HO·) to the degradation of isolated carbohydrates (Grootveld *et al.*, 1991; Schiller *et al.*, 1994), articular cartilage (Schiller *et al.*, 1998; Schiller *et al.*, 1995a) and synovial fluid (Schiller *et al.*, 1996;) has been intensively studied by high-resolution NMR spectroscopy to clarify what resonances may serve as "markers" for cartilage degradation.

The sensitivity of NMR to detect degradation products of cartilage is markedly enhanced by the presence of relatively mobile N-acetyl groups in cartilage polysaccharides. If degradation of cartilage occurs, the detection of resolved N-acetyl
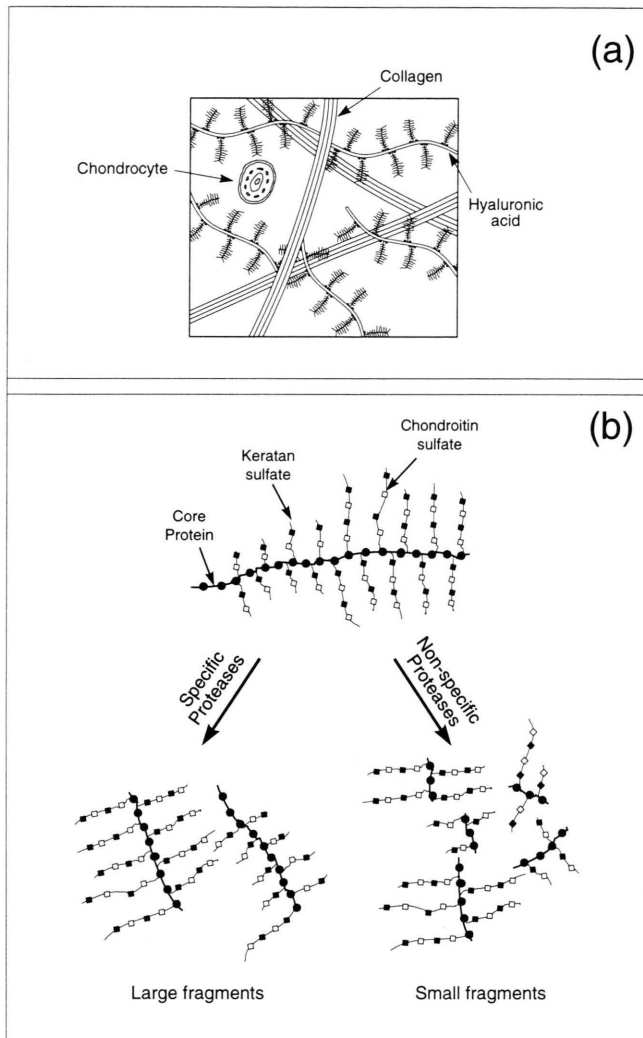
Fig. 1. Schematic picture of the organization of the extracellular matrix (a) and schematic representation of the action of proteases with different selectivities on cartilage proteoglycans (b). The more cleavage points one protease induces, i.e. the less selective it is, the smaller breakdown products are detectable in the supernatants of cartilage (●, proteins; □, GlcUA; ■ GalNAc-6-sulfate).

groups in comparison to the broad resonances of native cartilage polysaccharides becomes possible (Williamson *et al.*, 1989; Schiller *et al.*, 1996).

Because of the high selectivity of saccharidases like testicular hyaluronidase (Albert *et al.*, 1993) and especially chondroitinase ABC (Sugahara *et al.*, 1996), which produces only a single product from chondroitin sulfate and hyaluronic acid, saccharidase digestion products have been exhaustively characterized by NMR spectroscopy. In contrast, the action of proteases on cartilage yields a large variety of degradation products. Thus, proteolytic digestions of cartilage have been mainly characterized by changes in the dif-

fusion properties of water in cartilage (Burstein *et al.*, 1993) and by imaging methods (Bentley *et al.*, 1971) but only rarely by high resolution NMR spectroscopy.

On the other hand, proteases differ considerably in their selectivity: Since all carbohydrates of cartilage are linked to a central core protein (Fig. 1b), the different selectivities of proteolytic enzymes should be clearly reflected by the formation of digestion products with different molecular weights. Enzymes with lower selectivity produce more and smaller fragmentation products resulting in more intense N-acetyl resonances from the more mobile carbohydrates attached to the fragment peptides,

although the polysaccharides themselves are not damaged by proteases.

Thus, the aim of the present work is to use NMR to characterize the differences in the supernatants obtained after enzymatic digestion of articular cartilage by various proteases.

Additional analysis by matrix-assisted laser desorption and ionization (MALDI-TOF) mass spectrometry (Karas *et al.*, 1989; Hillenkamp *et al.*, 1991) is also performed.

Materials and Methods

Chemicals

Chemicals for buffer preparation (NaCl, Na_2HPO_4 and KH_2PO_4), for NMR spectroscopy (deuterated water with an isotopic purity of 99.95% and 3-(trimethylsilyl)-propane-1-sulfonate (TSP)), and for MALDI experiments (2,5-dihydroxy-benzoic acid (DHB), sinapinic acid, α-cyano-hydroxy-cinnamic acid, ethanol, acetonitrile and trifluoroacetic acid)) were of analytical grade (Fluka Feinchemikalien GmbH, Germany) and used without further purification.

The enzymes were purchased from Fluka and used without further purification as solutions containing one enzymic unit (1U) per milliliter. Whereas trypsin is a very specific enzyme, cleaving a protein only at the C-terminus of a lysine or arginine residue, collagenase and papain are by far less selective. These enzyme solutions were prepared immediately prior to use in 50 mM phosphate buffer containing 154 mM NaCl.

Collagen type II from bovine achilles tendon was obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany) and used as supplied.

Cartilage preparation

Fresh pig articular cartilage was obtained from juvenile animals (about 12 months old and without any traces of disease) within a few hours after slaughter. After bone was removed, cartilage was cut into small pieces, frozen at −196 °C in liquid nitrogen and finely minced in a portland mortar. Cartilages from different animals were combined in order to eliminate biological variation as a source of differences between the samples used in subsequent experiments (Schiller *et al.*, 1995b, Schiller *et al.*, 1995c).

Incubation conditions

Samples containing 0.5 g (wet weight) cartilage in 2.0 ml buffer (50 mM/l phosphate, 154 mmol/l NaCl, pH 7.4) containing a given enzyme were mixed in small beakers and incubated at 37 °C in a water bath for two hours. Control incubations were performed without added enzyme. Although collagenase and papain are known to be Ca^{2+}- and thiol-dependent, respectively, an addition of both ($CaCl_2$ and cysteine) was not found to enhance markedly enzymatic activity. It is assumed that from the cartilage tissue a sufficient amount of calcium ions and thiols are released. Thus, no external additions are required.

Subsequent to incubation, solutions were centrifuged for 10 min at 5000 rpm to remove insoluble material. The resulting clear, slightly yellow supernatants were analysed by ^1H NMR spectroscopy. For ^{13}C-NMR spectroscopy, samples were incubated for 12 hours to obtain higher concentrations of degradation products. Enzymatic digestion was stopped by the addition of a small quantity of EDTA and freezing the sample at −78 °C until measurement. Although EDTA does not terminate papain activity, no more activity of collagenase as well as of papain could be detected subsequent to freezing.

Incubations were also performed on 0.5 g (dry wt.) samples of commercial collagen II. Since native collagen is absolutely insoluble, resonances in the corresponding supernatant clearly indicate a degradation of collagen, i.e. a denaturation of collagen fibrils.

NMR measurements

a) ^1H-NMR

Proton-NMR measurements were conducted on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^1H. All spectra were recorded at ambient temperature (293 K) since this temperature was found to give reasonable resolution while avoiding heat-induced changes in the sample, which may occur at elevated temperatures (Schiller *et al.*, 1995c).

Typically, 0.40 ml supernatant from the incubation experiments was placed in a 5 mm diameter NMR tube and 50 µl of D_2O was added to provide a field frequency lock. The intense water signal

and broad resonances arising from proteins and polymeric carbohydrates of cartilage were suppressed by a combination of the Hahn spin-echo sequence (Bell *et al.*, 1988) and the application of presaturation on the water resonance frequency. Typically, 128 transients were acquired using the Hahn spin-echo sequence [90°-τ-180°-τ-collect] with τ = 60 ms and a repetition time of 8 seconds to allow full spin-lattice (T_1) relaxation of the protons in the sample.

Conventional pulse-acquire spectra were obtained using the same parameters. All spectra were recorded with a spectral width of 4000 Hz (13 ppm) and 16K data points. No window functions were used prior to Fourier transformation.

Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulfonate (TSP) at a final concentration of 500 μM (Schiller *et al.*, 1995a). Undeuterated TSP could be used in the present investigation since its resonances do not cover a spectral region of interest. Resonances were identified by their known chemical shifts (Agar *et al.*, 1991) and by their subsequent enhancement after addition of a small amount of the corresponding pure compound.

b) ^{13}C-NMR spectroscopy

Partially relaxed ^{13}C-NMR spectra were obtained at 75.47 MHz on the same spectrometer as described above. Data were recorded with a spectral width of 15600 Hz, 16K time-domain data points with a flip angle of 45° (2 μs pulse) and a repetition time of 2 s. Usually 8K transients were accumulated with WALTZ-16 ^{1}H decoupling. All free induction decays were processed with a 5 Hz line-broadening to enhance the signal to noise ratio.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager biospectrometry workstation using standard software (PerSeptive Biosystems, Framingham, UK). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage used was 20 kV (reflector mode) or 25 kV (linear mode). Pressure in the ion chamber was maintained between 1×10^{-7} and 4×10^{-7} Torr. Laser strength was kept about 10 percent over

threshold setting to obtain the best signal to noise ratio. Data from 128 single "shots" from the nitrogen laser (337 nm) were averaged for each mass spectrum. Samples were prepared by mixing 1 μl of supernatant with 1 μl matrix solution (50 mM 2,5-dihydroxy-benzoic acid (DHB) in a mixture of acetonitrile and water (70:30/v:v) containing 0.1% trifluoroacetic acid) directly on the sample plate. To improve crystallization, all samples were subsequently treated with 1 μl ethanol and allowed to recrystallize.

Results and Discussion

Cartilage consists of a complex network of different polysaccharides and proteins (mainly collagen II), which are not extractable from the tissue by buffer. Due to the metabolic activity of cartilage cells (chondrocytes), some physiologically relevant molecules are also detectable in cartilage supernatants (Agar *et al.*, 1991). Fig. 2 shows typical spin-echo (a) and pulse-acquired (b) ^{1}H-NMR
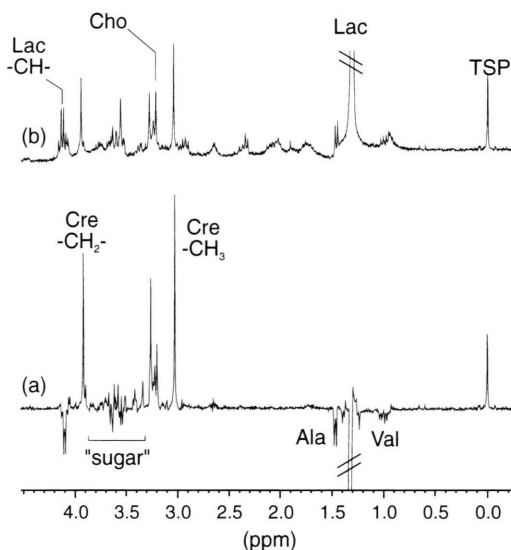

Fig. 2. ^{1}H-NMR spectra of pig articular cartilage (0.5 g wet weight) after incubation in 2 ml 50 mM phosphate buffer containing 154 mM NaCl at 37 °C for two hours. (a) was recorded using the Hahn spin-echo sequence with a delay τ=60 ms resulting in a turning of phases of coupled spin-systems, whereas (b) was obtained under analogous conditions as single pulse spectrum (cut off for clarity due to its high intensity).

Abbreviations used in peak assignments: Lac, lactate; Ala, alanine; Cre, creatine; Val, valine; Cho, choline; TSP (3-(trimethylsilyl)-propane-1-sulfonate).

spectra of the supernatants of pig articular cartilage incubated for 2 h in phosphate buffer (control). These different experimental conditions were used to determine the extent of a release of polymeric compounds on buffer extraction.

Most of the resonances in the spin-echo (TE~120 ms) spectra of cartilage supernatants derive from low-molecular weight metabolites of cells (Agar *et al.*, 1991). Spectra are clearly dominated by the lactate resonance at 1.31 ppm (Reiman *et al.*, 1990). The following signal assignments have been made: Creatine (3.90 and 3.00 ppm), trimethyl ammonium groups in metabolites such as phosphatidylcholine, glycerine-phosphatidylcholine, betaine (3.19 and 3.25 ppm), alanine (1.46 ppm), valine (0.93/0.97 ppm). Low-molecular carbohydrates (3.3–3.8 ppm) are also present in concentrations similar to other compounds.

In contrast, the pulse-acquire spectrum (2b) exhibits a number of additional, complex multiplets and weak resonances with considerable line widths, indicating small amounts of higher-molecular-weight species. Although large amounts of polymers are present in cartilage, polymers are not detectable in significant amounts in the control supernatant because they are caught in the gel structure of cartilage.

Fig. 3 compares NMR spectra of cartilage supernatants after incubation for two hours with pure buffer (a) and in the presence of different proteases (b-d). Pulse acquire spectra were examined in order to detect signals from high-molecular-weight species. In comparison to the control sample (buffer, 3a), additional resonances appear in the spectra 3b-3d at about 0.85, 2.02 and 3.55 ppm when cartilage is enzymatically digested, depending on the enzyme used. For example, only digestion with papain results in two resonances centred at 2.005 and 2.020 ppm which can be attributed to the N-acetyl groups of sulfated polysaccharides, still attached to peptide fragments which have been released into the supernatant (Schiller *et al.*, 1995a; Welti *et al.*, 1979). In addition, a broad signal band centred near 3.70 ppm corresponds to the less mobile ring C-H protons in oligosaccharides.

Papain does not act on collagen but mainly on the core protein of the cartilage proteoglycans. In spectrum 3d an intensity enhancement of the alanine resonance (1.46 ppm) and the valine resonances (0.97/0.93 ppm) is clearly detectable.
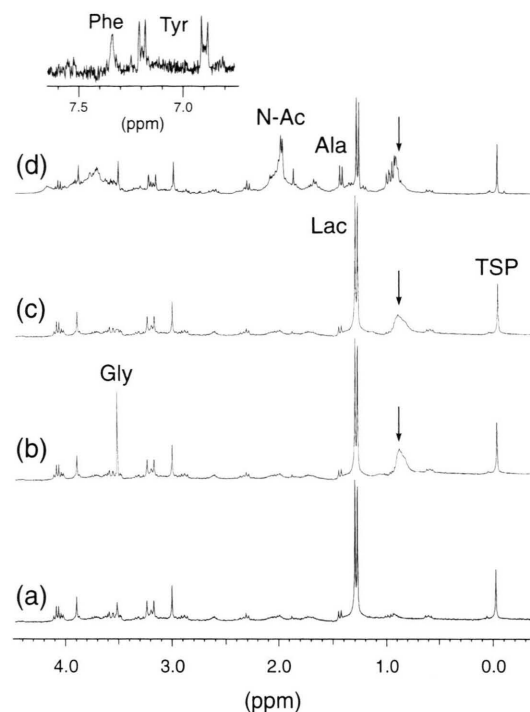

Fig. 3. ^{1}H-NMR single-pulse spectra of pig articular cartilage (0.5 g wet weight) after incubation in 2 ml 50 mM phosphate buffer containing 154 mM NaCl at 37 °C for two hours in the presence of different proteolytic enzymes. a, pure buffer; b, collagenase; c, trypsin; d, papain. Abbreviations used in peak assignments: Lac, lactate; Ala, alanine; Gly, glycine; N Ac, mobile N-acetyl groups of cartilage carbohydrates; TSP (3-(trimethylsilyl)-propane-1 sulfonate). Signals arising from collagen fragmentation are marked with an arrow.
The insert in (d) shows a part of the low-field region of the spectrum of papain treated cartilage. Abbreviations used: Tyr, tyrosine; Phe, phenylalanine.

Whereas alanine might also result from collagen degradation, valine is not present in high enough amounts in collagen to result in such a strong resonance. The same holds for the low-field region of the NMR spectrum recorded upon papain digestion (3d). Both doublets at 6.88 and 7.19 ppm correspond to the para substituted ring of tyrosine (AA'XX' spin system), whereas the resonance at 7.34 ppm corresponds to the phenyl protons of phenylalanine. Therefore, from these data papain must be regarded as the most effective enzyme for the digestion of non-collagenous proteins of cartilage. Due to its low specifity, conferring a large number of cleavage points, also rather rare amino

acids such as the aromatic amino acids are detectable in cartilage supernatants.

The treatment of cartilage with any of the three proteases (b-d) results in a broad resonance at ca. 0.85 ppm, which might correspond to the relatively mobile methyl groups (e.g. in Leu, Val, and Ile) in (denatured) collagen degradation products (Agar *et al.*, 1991).

In supernatants of cartilage samples treated with collagenase (3b) an intense singlet at 3.55 ppm is observed after papain treatment. This resonance obviously results from glycine (Agar *et al.*, 1991), which is present in constant, high proportions (about 33%) in all cartilage collagens. The glycine singlet is easily detectable by NMR (it contains only a single kind of protons) and provides a reliable indicator of collagen degradation.
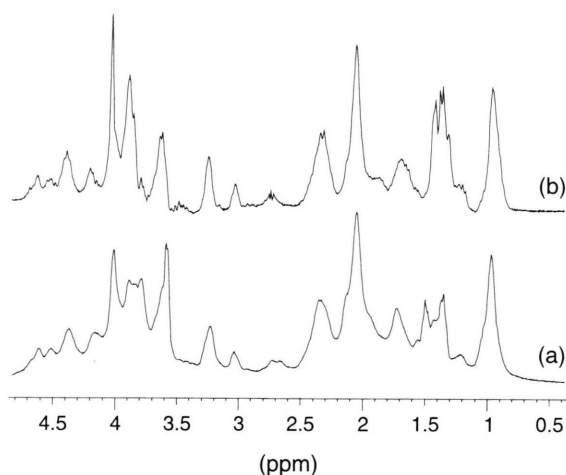

Fig. 4. ^{1}H-NMR pulse-acquire spectra of pig articular cartilage (a) and commercially available, native collagen type II (b) after enzymatic digestion with 1 mg/ml collagenase for 12 hours.

To enhance the effects of collagenase, incubation times were extended to 12 hours. In Fig. 4 the NMR spectra of exhaustive collagenase digestion of cartilage (a) and commercial collagen (b) are shown. In comparison to Fig. 3 longer digestion results in much higher intensity for broad signals (i.e. high-molecular components) over the whole spectral region. On the other hand, exhaustive enzymatic degradation of cartilage also enhances viscosity of samples, which conferres considerable broadening of lines. It is obvious that the spectrum of collagen supernatant closely resembles the spectrum of cartilage supernatants and the reso-

nances observed are indicative of oligopeptides and to a smaller extent oligosaccharides. Information on collagen degradation is more difficult to obtain because of the rigidity of the collagen polypeptide and the nearly complete absence of N-acetyl groups in the attached disaccharide. However, solid state ^{13}C- (Saito and Yokoi, 1992) and ^{15}N-NMR (Naito *et al.*, 1994) studies have been performed with isotopically enriched collagen but studies on supernatants of collagen subsequent to enzymatic treatment are rare.

Additional information concerning the composition of the mixture was obtained with ^{13}C NMR (Fig. 5). In the carbon NMR spectra of cartilage supernatants obtained after 2 h incubation with buffer (a, control) or with collagenase (b) only a few resonances can be detected. In the control supernatant (a) only lactate was observed (187.1, 73.0, and 24.7 ppm) (Kalinowski *et al.*, 1984) while collagenase digestion (b) resulted in two additional resonances for glycine (177.0, and 45.8 ppm) (Kalinowski *et al.*, 1984). Thus, after 2 h incubations only the two substances at highest concentration (Lac, Gly) were detected by ^{13}C NMR, in agreement with ^{1}H NMR results (3a, 3b).

Figs. 5c and 5d show the supernatant spectra obtained after 12 h incubations of cartilage with collagenase and papain, respectively. For comparison spectrum (5e) was obtained from a collagenase digest of commercial collagen type II, and (5f) from a reference solution of chondroitin sulfate (mixture of the 4- and 6-isomers) in phosphate buffer (Bociek *et al.*, 1980). It is evident that collagenase treatment of pure collagen (5e) or cartilage (5c) results in very similar spectra which differ significantly from the spectrum of chondroitin sulfate (5f), which in turn closely resembles the papain digest (5d).

Since only four amino acids (glycine, proline, alanine and hydroxyproline) constitute two thirds of the total amino acid composition of cartilage collagen, these amino acids can be clearly detected in the spectra obtained from collagenase digests. Their chemical structures and the resulting ^{13}C-NMR shifts are given at the bottom of Fig. 5. However, signals from other amino acids are also observed, and the presence of a complex mixture of oligopeptides is suggested by many resonances from carboxylate (COO$^-$) and carbonyl (C=O) groups in the range 170–185 ppm. The papain di-
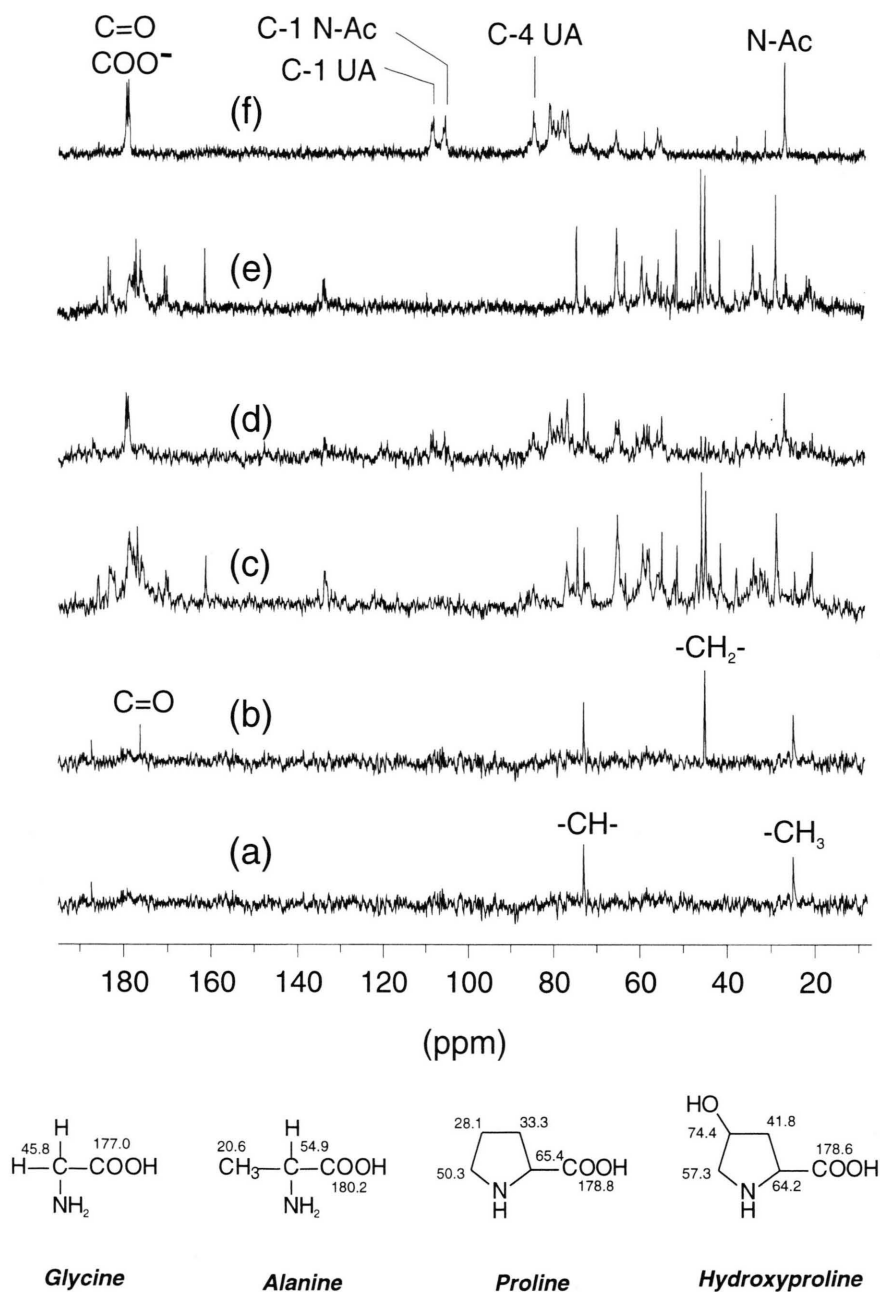
Fig. 5. ^{13}C NMR spectra of the supernatants of articular cartilage upon two hours incubation with pure phosphate buffer (a) and 1 mg/ml collagenase for two hours (b). In c-e an incubation period of 12 hours was chosen. (c) cartilage + 1 mg/ml collagenase, (d) cartilage + 1 mg/ml papain, (e) commercially available, native collagen type II + 1 mg/ml collagenase. In (f) the corresponding spectrum of a pure chondroitin sulfate solution (50 mM) in phosphate buffer is shown. Abbreviations used in peak assignments: UA, uronic acid; N-Ac, N-acetylgalactosamine.
The ^{13}C chemical shifts of some relevant amino acids of collagen are given at the bottom of the figure.

gest of cartilage gives quite a different spectrum and exhibits predominantly the signals of carbohydrates. Signals characteristic of Gal-NAc (C1, CH$_3$ and C=O of N-Ac) and of Glc-UA (C1, C4, carbonyl) of chondroitin sulfate (Bociek et al., 1980) were detected in the papain digest spectrum (5d) but were absent in the spectra obtained after collagenase digestion. Whereas Torchia et al. have shown by means of ^{13}C NMR that papain releases also cartilage peptides (Torchia et al., 1977) upon papain digestion, peptide signals were barely detectable in cartilage supernatants in our studies (compare Figs. 5c and 5d).

The recently developed matrix-assisted laser desorption and ionization (MALDI) mass spectrometry was also applied as an additional analytical method. Fig. 6 shows the high-mass (a) and low-mass (b) regions of the positive ion MALDI-mass spectra of the collagenase digest of cartilage. High-mass components were detectable with m/z 4760, 3990 and 2947 Da in supernatants of cartilage as well as in collagen (data not shown). All peaks are relatively broad due to the association of sodium and potassium ions to the negatively charged groups of the proteins. Nevertheless, the presence of these peaks is consistent with the NMR spectra of Fig. 4a and 5c, i.e. the presence of large peptide fragments from the proteolytic breakdown of cartilage collagens.

In Fig. 6b there are peaks corresponding to the molecular weights of glycine (75.2), alanine (90.2), proline (116.7) and hydroxyproline (131.3). The same amino acids were also observed in the corresponding ^{13}C NMR spectrum (Fig 5c and 5e). The more intense and broader peaks at 155.2 (M+H), 137.1 (M-H$_2$O), and 110.3 (M-CO$_2$) in 6b arise from the dihydroxybenzoic acid (DHB, MW= 154.12) matrix used. Additionally, a very small peak is also detectable at 287.2 (data not shown), which is consistent with the tripeptide Gly-Pro-Hyp frequently occurring in the collagen polypeptide (Saito et al., 1984).

Summarizing, high-resolution NMR spectroscopy offers the possibility to differentiate between the effects of various proteases, and these data correlate very well with other experimental data.
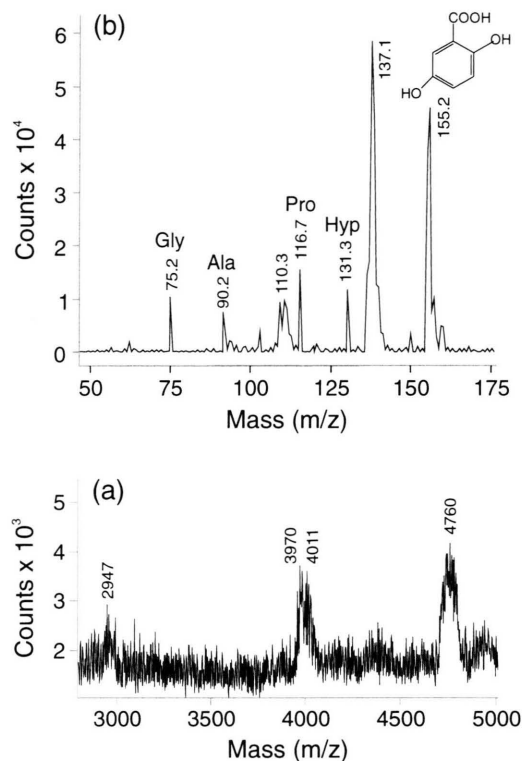

Fig. 6. Positive ion MALDI spectrum of the supernatant of articular cartilage upon collagenase digestion. (a) shows the high molecular mass part, whereas (b) shows the low-molecular mass region. In both cases 2,5-dihydroxy-benzoic acid was used as matrix material. Amino acids were identified by their characteristic molecular masses.

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