*Journal of Enzyme Inhibition and Medicinal Chemistry,* 2002 VOL. 17 (3), pp. 183–186





# Inhibition of Hyaluronidases and Chondroitinases by Fatty Acids

KEITAROU SUZUKI, YOSHIKAZU TERASAKI and MASARU UYEDA*

*Laboratory of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan*

*(Received 20 March 2002)*

**The inhibitory effects of various fatty acids on three hyaluronidases (h-ST, h-SH and h-SD) and four chondroitinases (c-ABC, c-B, c-ACI and c-ACII) were examined, and their structure–activity relationships and mechanism of action were studied. The fatty acids used in this experiment showed various inhibitory activities against the enzymes. None of the fatty acids did not inhibit h-ST and h-SH. The saturated fatty acids ($C_{10:0}$ to $C_{22:0}$) showed very weak or no inhibition against h-SD, c-ABC, c-B, c-ACI and c-ACII but the unsaturated fatty acids ($C_{14:1}$ to $C_{24:1}$) with one double bond strongly inhibited the enzymes, and the inhibitory potency increased with increase in carbon chain length of the fatty acids. In contrast, the increase in number of double bonds caused a decrease in inhibitory potency against the enzymes. The position of the double bond and the stereochemistry of the *cis-trans* form of oleic acid ($C_{18:1}$) did not influence the inhibitory potency against the enzymes. Carboxyl and hydroxyl groups in the fatty acid molecule were concerned in the inhibition of c-ACI. Among the fatty acids, eicosatrienoic acid ($C_{20:3}$) generally inhibited h-SD, c-ABC, c-B and c-ACI, and nervonic acid ($C_{24:1}$) was a potent inhibitor of c-ACII, and the fatty acids inhibited the enzymes in a noncompetitive manner.**

*Keywords*: Fatty acid; Hyaluronidase; Chondroitinase; Inhibition

## INTRODUCTION

Hyaluronic acid is a glycosaminoglycan in the extracellular matrix, and its structure is a straight chain polymer of disaccharides consisting of N-acetyl-D-glucosamine and D-glucuronic acid. Hyaluronidases degrade hyaluronic acid to low molecular saccharides and the enzymes have been classified on the basis of substrate specificity and reaction mechanism.[1] The enzymes are widely distributed in animal tissues and microorganisms. Some of the hyaluronidases are components in the venoms produced by various organisms such as bacteria, bees, spiders, scorpions, snakes and lizards.[2] The bacterial hyaluronidases are virulent factors associated with adhesion, invasion and penetration of animal tissues, and the enzymes act as facilitating factors for diffusion of other components in the venom.[3] Therefore, an inhibitor of hyaluronidase would be useful for the prevention of infection and poisoning. It is known that polysaccharides (alginic acids,[4] pectins,[5] glycosaminoglycans[6]), flavonoids,[7] glycyrrhizin,[8] and saponins[9] inhibit hyaluronidases, but there are no specific inhibitors against the enzymes. In a search for inhibitors, we have screened various microorganisms and found that the fatty acids produced by streptomycetes inhibited a hyaluronidase from *Streptococcus dysgalactiae* (h-SD).[10] Consequently, we have examined the inhibitory activities of various fatty acids against three kinds of hyaluronidases. We have also examined their inhibitory effect on four chondroitinases which have similar properties to the hyaluronidases.

## MATERIALS AND METHODS

### Enzymes and Substrates

Hyaluronidase ST (h-ST, EC 3.2.1.35) from sheep testes,[12] hyaluronidase SH (h-SH, EC 4.2.2.1) from *Streptomyces hyalurolytics*,[11] hyaluronidase SD (h-SD, EC 4.2.2) from *Streptococcus dysgalactiae*,[10] chondroitinase ABC (c-ABC, EC 4.2.2.4) from

*Corresponding author. Tel./fax: +81-96-371-4323. E-mail: uyeda@gpo.kumamoto-u.ac.jp

ISSN 1475-6366 print/ISSN 1475-6374 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/14756360290032930

*Proteus vulgaris*,[15] chondroitinase B (c-B, no EC number) from *Flavobacterium heparinum*,[16] chondroitinase ACI (c-ACI, EC 4.2.2.5) from *Flavobacterium heparinum*,[13] chondroitinase ACII (c-ACII, EC 4.2.2.5) from *Arthrobacter aurescens*,[14] hyaluronic acid from pig skin, dermatan sulfate (chondroitin sulfate B) from pig skin, chondroitin sulfate C from shark cartilage were obtained from Seikagaku Kogyo Co. (Tokyo).

### Fatty Acids

Saturated fatty acids ($C_{10:0}$ to $C_{22:0}$), *cis*-unsaturated fatty acids ($C_{14:1}$ to $C_{24:1}$), linoleic acid, linolenic acid, elaidic acid, ricinoleic acid and methyloleate were obtained from Funakoshi Co. (Tokyo). *Cis*-unsaturated fatty acids of twenty carbon atoms ($C_{20:2}$ to $C_{20:5}$) were obtained from Cayman Chemical Co. (Ann Arbor).

### Enzyme Reactions

H-SH, h-SD, c-ABC, c-B, c-ACI and c-ACII are lyases (eliminases) which catalyze the eliminative cleavage of hexosaminide linkages in the substrate molecule, yielding the disaccharides (tetra- and hexasaccharides for h-SH) with unsaturated glucuronic acid at the non-reducing ends.[11–14] Activities of the enzymes were measured based on the increase in the absorbance at 232 nm.[17] The reaction mixture (200 μl) consisting of each enzyme, 50 mM buffer and 500 μg substrate (hyaluronic acid for h-SH and h-SD, chondroitin sulfate C for c-ABC, c-ACI and c-ACII, dermatan sulfate for c-B) was incubated for 20 min at 37°C (60°C for h-SH, 30°C for c-B). The reaction was terminated with 600 μl of 0.04N HCl, and UV absorption of the mixture was measured at 232 nm. The reaction buffers used were phosphate (pH 6.2) for h-SH and h-SD, Tris–HCl (pH 8.0) for c-ABC and c-B, Tris–HCl (pH 7.3) for ACI, and acetate (pH 6.0) for c-ACII. H-ST is a hydrolase which catalyzes the hydrolytic cleavage of hexosaminide linkages in hyaluronic acid molecule, yielding the saturated tetra- and oligosaccharides with glucuronic acid at the non-reducing ends.[10] The activity of h-ST was measured based on the increase in the reducing power of tetrasaccharides released from hyaluronic acid by the enzyme. The reaction mixture (500 μl) consisting of the enzyme, 20 mM phosphate buffer (pH 6.0) and 500 μg hyaluronic acid was incubated for 20 min at 37°C, and the reaction was terminated by addition of 200 μl of 0.4 M boric acid–KOH buffer (pH 9.0) containing 0.05N NaOH and 1% SDS. After heating at 100°C for 2 min, the reducing power of the mixture was measured by the modified Morgan–Elson method.[18] The inhibitory activity ($IC_{50}$) was defined as the amount of fatty acid that reduced enzyme activity by 50%.

## RESULTS AND DISCUSSION

### Inhibitory Effects of Saturated Fatty Acids

Inhibitory effects of some saturated fatty acids ($C_{10:0}$ to $C_{22:0}$) on hyaluronidases (h-ST, h-SH and h-SD) and chondroitinases (c-ABC, c-B, c-ACI and c-ACII) were examined. As shown in Table I, the three hyaluronidases and four chondroitinases were either not or very weakly inhibited by these fatty acids. The saturated fatty acids did not have high inhibitory potency against the glycosaminoglycanases such as hyaluronidases, chondroitinases and heparinases. In relation to the above results, there are some reports that saturated fatty acids do not influence the activities of various enzymes such as methyltransferase, topoisomerase, ATPase, reductase etc.[19–22]

### Inhibitory Effects of *Cis*-unsaturated Fatty Acids

As shown in Table II, *cis*-unsaturated fatty acids ($C_{14:1}$ to $C_{24:1}$) containing one double bond in the molecule inhibited h-SD and the four chondroitinases which are able to release the disaccharides from substrates as end products, however, the fatty acids did not inhibit h-ST and h-SH which release tetra- and oligosaccharides without releasing

TABLE I Inhibitory effects of saturated fatty acids on hyaluronidases and chondroitinases

| Fatty acid | No. of carbon atoms and double bond | Inhibition ($IC_{50}$, μM)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | h-ST | h-SH | h-SD | c-ABC | c-B | c-ACI | c-ACII |
| Capric acid | 10:0 | >500 | >500 | >500 | 298 | >500 | >500 | >500 |
| Lauric acid | 12:0 | >500 | >500 | >500 | 265 | 384 | >500 | >500 |
| Myristic acid | 14:0 | >500 | >500 | >500 | 144 | 352 | >500 | 483 |
| Palmitic acid | 16:0 | >500 | >500 | >500 | 154 | 352 | >500 | 299 |
| Stearic acid | 18:0 | >500 | >500 | >500 | 171 | 460 | >500 | 301 |
| Arachidic acid | 20:0 | >500 | >500 | >500 | 174 | 491 | >500 | 348 |
| Behenic acid | 22:0 | >500 | >500 | >500 | 183 | >500 | >500 | 458 |

* Values represent the means obtained from three independent experiments, and each value was within the range of ±5%.

TABLE II Inhibitory effects of *cis*-unsaturated fatty acids containing one double bond on hyaluronidases and chondroitinases

| Fatty acid | No. of carbon atoms and double bonds | Position of double bond | Inhibition ($IC_{50}$, μM)* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | h-ST | h-SH | h-SD | c-ABC | c-B | c-ACI | c-ACII |
| Myristoleic acid | 14:1 | 9-*cis* | >500 | >500 | 172 | 6 | 37 | >500 | 417 |
| Palmitoleic acid | 16:1 | 9-*cis* | >500 | >500 | 37 | 5 | 31 | 175 | 294 |
| Oleic acid | 18:1 | 9-*cis* | >500 | >500 | 31 | 2 | 14 | 63 | 251 |
| Eicosanoic acid | 20:1 | 11-*cis* | >500 | >500 | 16 | 2 | 12 | 47 | 140 |
| Docosenoic acid | 22:1 | 13-*cis* | >500 | >500 | 16 | 1 | 11 | 54 | 78 |
| Nervonic acid | 24:1 | 15-*cis* | >500 | >500 | 17 | 1 | 11 | 77 | 49 |

*Values represent the means obtained from three independent experiments, and each value was within the range of ±5%.



disaccharides. It was clear that the unsaturated fatty acids selectively inhibit the glycosaminoglycanases which are able to release the disaccharides from hyaluronates and chondroitins. Moreover, the inhibitory potency of the fatty acids increased with increase in carbon chain length and reached a maxima at around 22 carbon atoms ($C_{20:1}$ for h-SD and c-ACI, $C_{22:1}$ for c-ABC and c-B, $C_{24:1}$ for c-ACII). The results suggested that the existence of the double bond in a fatty acid molecule is an essential factor for the inhibition and that carbon chain length influences inhibitory potency.

Inhibitory effects of the number of double bond on the enzymes were examined for *cis*-unsaturated fatty acids of $C_{18}$ and $C_{20}$. As shown in Table III, the fatty acids showed no inhibition against h-ST and h-SH, and the inhibitory potency against the other enzymes generally decreased with the increase in the number of double bond in the fatty acid.

## Inhibitory Effects of Isomers of Oleic Acid

The inhibitory effects of isomers of oleic acid ($C_{18:1}$) on hyaluronidases and chondroitinases were examined. As shown in Table IV, the isomers showed little difference in their inhibitory activities against the enzymes except for c-ACI. The inhibition by methyloleate and ricinoleic acid of c-ACI remarkably decreased compared to that of oleic acid. The results suggest that the carboxyl group in the fatty acid molecule may be important in the interaction with the c-ACI molecule. The position of the double bond and the *cis-trans* form of the fatty acid molecule did not greatly influence the inhibition.

## *Ki* Values of Eicosatrienoic Acid and Nervonic Acid

Eicosatrienoic acid ($C_{20:3}$) showed relatively strong inhibition against h-SD, c-ABC, c-B and c-ACI, and nervonic acid ($C_{24:1}$) was also a potent inhibitor of c-ACII. The type of inhibition was determined by Lineweaver–Burk reciprocal plots[23] of the substrate concentrations against the activities of the enzymes in the presence or absence of the fatty acids. As shown in Fig. 1, the enzyme activities of h-SD, c-ABC, c-B and c-ACI were noncompetitively inhibited by eicosatrienoic acid. c-ACII was also inhibited by nervonic acid in a noncompetitive manner. From the results, the fatty acids were considered to bind to a different site from the binding site of the substrates on the enzyme. The inhibitory potency (value of $Ki/Km$) of eicosatrienoic acid was in the following order: c–ABC(0.5) > c–B(2.3) > c–ACI(2.6) > h–SD(8.1), and eicosatrienoic acid and nervonic acid showed the same inhibitory potency (0.5) against c-ABC and c-ACII, respectively.

The fatty acids showed various inhibition of glycosaminoglycanases such as hyaluronidases and chondroitinases. Our data suggests that the unsaturated fatty acids selectively inhibit the glycosaminoglycanases which are able to release the disaccharides from hyaluronates and chondroitins,

TABLE III Inhibitory effects of the number of double bond in *cis*-unsaturated fatty acids on hyaluronidases and chondroitinases

| Fatty acid | No. of carbon atoms and double bonds | Position of double bonds | Inhibition ($IC_{50}$, μM)* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | h-ST | h-SH | h-SD | c-ABC | c-B | c-ACI | c-ACII |
| Oleic acid | 18:1 | 9-*cis* | >500 | >500 | 31 | 2 | 14 | 63 | 251 |
| Linoleic acid | 18:1 | 9,12-*cis* | >500 | >500 | 32 | 2 | 15 | 106 | 261 |
| Linolenic acid | 18:1 | 9,12,15-*cis* | >500 | >500 | 36 | 2 | 15 | 148 | 348 |
| Eicosanic acid | 20:1 | 11-*cis* | >500 | >500 | 15 | 2 | 12 | 47 | 140 |
| Eicosadienoic acid | 20:2 | 11,14-*cis* | >500 | >500 | 15 | 5 | 30 | 32 | 147 |
| Eicosatrienoic acid | 20:3 | 8,11,14-*cis* | >500 | >500 | 12 | 5 | 33 | 25 | 293 |
| Eicosatetraenoic acid | 20:4 | 5,8,11,14-*cis* | >500 | >500 | 18 | 23 | 62 | 102 | 298 |
| Eicosapentaenoic acid | 20:5 | 5,8,11,14,17-*cis* | >500 | >500 | 21 | 27 | 83 | 148 | 355 |

*Values represent the means obtained from three independent experiments, and each value was within the range of ±5%.

TABLE IV Inhibitory effects of isomers of oleic acid on hyaluronidases and chondroitinases

| Fatty acid | No. of carbon atoms and double bonds | Position of double bond | Inhibition ($IC_{50}$, μM)* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | h-ST | h-SH | h-SD | c-ABC | c-B | c-ACI | c-ACII |
| Oleic acid | 18:1 | 9-*cis* | >500 | >500 | 31 | 2 | 14 | 63 | 251 |
| Petroselinic acid | 18:1 | 6-*cis* | >500 | >500 | 16 | 2 | 16 | 55 | >500 |
| Vaccenic acid | 18:1 | 11-*cis* | >500 | >500 | 18 | 2 | 14 | 32 | 270 |
| Elaidic acid | 18:1 | 9-*trans* | >500 | >500 | 40 | 1 | 14 | 79 | 278 |
| Methyloleate | 19:1 | 9-*cis* | >500 | >500 | 42 | 2 | 38 | >500 | >500 |
| Ricinoleic acid† | 18:1 | 9-*cis* | >500 | >500 | 60 | 2 | 25 | >500 | 387 |

*Values represent the means obtained from three independent experiments, and each value was within the range of ±5%. †12-Hydroxy oleic acid.

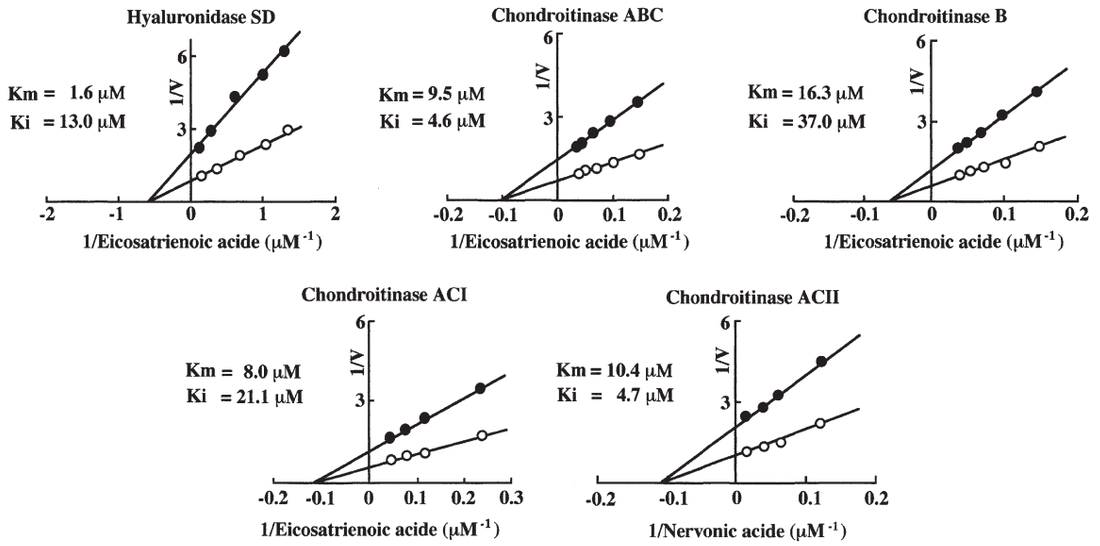


FIGURE 1 Lineweaver–Burk reciprocal plots of substrate concentrations against the activities of hyaluronidase (h-SD) and chondroitinases (c-ABC, c-B, c-ACI and c-ACII) with (•) and without (○) eicosatrienoic acid or nervonic acid. The concentrations of eicosatrienoic acid in the reaction mixture of h-SD, c-ABC, c-B and c-ACI were 15, 5, 35 and 25 μM, respectively. The concentration of nervonic acid for c-ACII was 50 μM. The substrates used were hyaluronic acid for h-SD, chondroitin sulfate C for c-ABC, ACI and ACII, and dermatan sulfate for c-AB. The conditions for assay of each enzyme are described in "Materials and Methods."

but do not inhibit the enzymes which release the tetra- and oligosaccharides as the end product. Fatty acids may be useful tools for studies connected with the glycosaminoglycanases.

## *References*


[1] Kreil, G. (1995) *Protein Sci.* **4**, 1666–1669.
[2] Frost, G.I., Csoka, T. and Stern, R. (1996) *Trends Glycosci. Glycotechnol.* **8**, 419–434.
[3] Hynes, W.L. and Walton, S.L. (2000) *FEMS Microbiol. Lett.* **183**, 201–207.
[4] Asada, M., Sugie, M., Inoue, M., Nakagomi, K., Hongo, S., Murata, K., Irie, S., Takeuchi, T., Tomizuka, N. and Oka, S. (1997) *Biosci. Biotechnol. Biochem.* **61**, 1030–1032.
[5] Sawabe, Y., Nakagomi, K., Iwagami, S., Suzuki, S. and Nakazawa, H. (1992) *Biochim. Biophys. Acta* **1137**, 274–278.
[6] Toida, T., Ogita, Y., Suzuki, A., Toyoda, H. and Imanari, T. (1999) *Arch. Biochem. Biophys.* **370**, 176–182.
[7] Kuppusamy, U.R., Khoo, H.E. and Das, N.P. (1990) *Biochem. Pharmacol.* **40**, 397–401.
[8] Furuya, T., Yamagata, S., Shimoyama, Y., Fujihara, M., Morishima, N. and Ohtsuki, K. (1997) *Biol. Pharm. Bull.* **20**, 973–977.
[9] Facino, R.M., Carini, M., Stefani, R., Aldini, G. and Saibene, L. (1995) *Arch. Pharm.* **328**, 720–724.
[10] Meyer, K. (1971) In: Boyer, P.D., ed, *The Enzymes* (Academic Press, New York) **Vol. 5**, pp 307–320.
[11] Ohya, T. and Kaneko, Y. (1970) *Biochim. Biophys. Acta* **198**, 607–609.
[12] Calvinho, L.F., Almeida, R.A. and Oliver, S.P. (1998) *Vet. Microbiol.* **61**, 93–110.
[13] Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K. and Suzuki, S. (1997) *J. Biol. Chem.* **272**, 9123–9130.
[14] Pojasek, K., Shriver, Z., Kiley, P., Venkataraman, G. and Sasisekharan, R. (2001) *Biochem. Biophys. Res. Commun.* **286**, 343–351.
[15] Jandik, K.A., Gu, K. and Linhardt, R.J. (1994) *Glycobiology* **4**, 289–294.
[16] Hiyama, K. (1976) *J. Biochem.* **80**, 1209–1214.
[17] Yamagata, T., Saito, H., Habuchi, O. and Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535.
[18] Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1995) *J. Biol. Chem.* **217**, 959–966.
[19] Suzuki, K., Nagao, K., Tokunaga, J., Katayama, N. and Uyeda, M. (1996) *J. Enz. Inhib.* **10**, 271–280.
[20] Suzuki, K., Shono, F., Kai, H., Uno, T. and Uyeda, M. (2000) *J. Enz. Inhib.* **15**, 357–366.
[21] Murakami, S., Murumatsu, M., Araki, H. and Otomo, S. (1994) *Res. Commun. Mol. Pathol. Pharmacol.* **85**, 57–66.
[22] Imamura, Y., Migita, T., Otagiri, M., Choshi, T. and Hibino, S. (1999) *J. Biochem.* **125**, 41–47.
[23] Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.