Free 4-Hydroxyproline Content in Serum of Bedridden Aged People Is Elevated Due to Fracture

Katsuya KOIKE,^a Yanmei LI,^a Miyuki SEO,^b Ikuo SAKURADA,^c Ko TEZUKA,^c and Kazuo UCHIKAWA^{*,d}

Biochemistry Laboratory, College of Pharmacy, Nihon University,^a 7-7-1 Narashino-dai, Funabashi, Chiba 274-8555, Japan, Yamato Clinic,^b 420 Odana, Yamato-son, Oshima-gun, Kagoshima 894-3106 Japan, Comet Co.,^c 1087-131 Kamiaso, Aso-ku, Kawasaki, Kanagawa 215-0021, Japan, and Medical Chemistry Laboratory, College of Pharmacy, Nihon University,^d 7-7-1 Narashino-dai, Funabashi, Chiba 274-8555, Japan.

Received July 1, 1999; Accepted September 30, 1999

Hydroxyproline (Hyp) content in biological fluids is used as a parameter of collagen catabolism, especially bone resorption. In this paper, we examined the relationship between aging and serum free 4-Hyp and proline (Pro) content using a newly introduced analytical technique which is chemiluminescence determination with electrogenerated tris (2,2'-bipyridine) ruthenium(III). Serum was collected from 50 bedridden aged people and 131 normal subjects living in Yamato-son, Amamioshima, Kagoshima. In the free 4-Hyp content of normal serum, the change in females is more gentle than that males with aging. The free 4-Hyp content of bedridden aged serum is significantly ($p < 0.05$) elevated compared to normal aged serum. Bedridden aged females and males also show levels significantly ($p < 0.05$) elevated in comparison to normal. These changes are due to bone resorption based on bedridden patients' atrophy. In bedridden aged people, free 4-Hyp of those with a fracture was significantly ($p < 0.05$) elevated than in people without a fracture. No relationship between low bone density and high free 4-Hyp in serum was observed. It seems that bone resorption or bedridden atrophy may be enhanced in bedridden aged people by a fracture. In free Pro content in serum, no significant change was observed. These results show that the free 4-Hyp content in serum is useful in the clinical or biological inspection of bone resorption, especially in aged people.

Key words 4-hydroxyproline; proline; chemiluminescence; tris-(2,2'-bipyridine)-ruthenium; serum; human

Hydroxyproline (Hyp) is a collagen protein specific imino acid, which is synthesized by the posttranslational hydroxylation of proline (Pro). Hyp content in biological fluids is used as a parameter of collagen catabolism, especially bone adsorption.¹⁾ It has been reported that the increase in Hyp and Pro content in biological fluids is strongly related to bone disease,^{2,3)} fracture,¹⁾ cancer,⁴⁻⁹⁾ and cirrhosis.¹⁾ Also, there have been reports of increased serum Hyp content in bedridden old people,¹⁾ although the total Hyp content in serum decreases during aging.¹⁰⁾

We had the chance to investigate free serum Hyp and Pro content as a part of a medical check of persons living in Yamato-son project (Amamioshima, Kagoshima, Japan) in 1998. In this paper, we examine the relationship between free 4-Hyp and Pro content in serum and aging using a newly introduced analytical technique.

MATERIALS AND METHODS

Reagents All chemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless noted otherwise.

Preparation of Analytical Serum Sample and Measurement of Bone Density Serum samples were collected from 50 bedridden old people (39 females and 11 males, aged 68 to 100) and 131 normal subjects (103 females and 28 males, aged 22 to 93) living in Yamato-son (Amamioshima, Kagoshima, Japan) from August to November in 1998, and serum was stored in -80°C until analysis. Three hundred microliters of serum was applied to a centrifugal filter device (Microcon®, YM-10, Millipore Co., Bedford, MA, U.S.A.) and was centrifuged (14000 rpm, 60 min, 15°C). The filtrate was used for free 4-Hyp and Pro analysis, directly. The bone

mineral density (BMD, g/cm^2) was measured at the radius and ulna located in the distal forearm by Osteometer DTX-200 (Lunar Co., Madison, WI, U.S.A.).

Hyp and Pro Analysis Free 4-Hyp and Pro analysis in human serum were done according to our previous paper¹¹⁻¹³⁾ using an HPLC system with an electrogenerated chemiluminescence detector. HPLC conditions were as follows: separation—eluent: 20 mM sodium acetate buffer (pH 4.62), 1 mM Cu^{2+} , 10 mM 1-octanesulfonate-acetonitrile (99 : 1, v/v), flow rate: 0.5 ml/min, column: CAPCELL PAK C18 (4.6×150 mm, Shiseido Co., Ltd., Tokyo, Japan), detection—reagent solution: 0.3 mM tris (2,2'-bipyridine) ruthenium(II) chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), 10 mM sulfuric acid, flow rate: 0.3 ml/min, electrochemiluminescence detector (Comet 2000, Comet Co., Ltd., Tokyo, Japan): controlled current oxidation as $80 \mu\text{A}$. The filtered serum was injected to the HPLC system, directly. Free 4-Hyp and Pro content in serum were analyzed using available authentic 4-Hyp (Sigma Chemical Co., St. Louis, MO, U.S.A.) and Pro (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard.

Statistics The values are expressed as mean $\pm$ S.E. Statistical processing was done by *U*-test (Mann-Whitney), unless noted otherwise. Differences with a probability of less than 5% were considered to be statistically significant ($p < 0.05$). Additionally, as a control against the bedridden aged people group, we employed some normal subjects (23 females and 8 males, aged 80 to 93).

RESULTS

Calibration curves plotted the peak area *versus* Hyp and Pro concentrations. The assay was linear in the concentration

* To whom correspondence should be addressed.

range of 0.27–10000 and 0.59–5000 nmol/ml serum for Hyp and Pro, respectively. The recovery was 101.5% (10 nmol/ml) for Hyp and 103.1% (50 nmol/ml) for Pro. The inter-assay coefficient of variation was less than 5% for both Hyp and Pro in linear concentration range. A representative chromatogram of a serum sample is shown in Fig. 1.

Changes in the concentration of free 4-Hyp and Pro in normal object serum with aging are shown in Fig. 2, with values of 13.7 ± 0.6 and 94.5 ± 3.1 $\mu\text{mol/l}$, respectively. No significant change was observed between female and male in 4-Hyp and Pro content.

The concentrations of free 4-Hyp and Pro in bedridden old and normal aged people serum are shown in Table 1. The free 4-Hyp content of bedridden aged people was significantly ($p < 0.05$) higher than normal aged people, by 19%. The free 4-Hyp content of female and male bedridden aged people was significantly ($p < 0.05$) higher than in normal aged people, by 25 and 15%, respectively. No significant change was observed in the Pro content or BMD value between any group, except the BMD of bedridden aged females.

The concentrations of free 4-Hyp and Pro and BMD value within or without fractured bedridden aged peoples' serum are shown in Table 2. Free 4-Hyp content of fractured bedrid-

den aged people was significantly ($p < 0.05$) higher than in those without fractures, by 30%. The free 4-Hyp content in females was also significantly ($p < 0.05$) higher than in those without fractures, by 29%. No significant change was observed in the Pro content or BMD value between any groups.

DISCUSSION

There are many analytical methods available to determine Hyp or Pro in biological fluids. Our newly introduced analytical technique, which is chemiluminescence determination with electrogenerated tris (2,2'-bipyridine) ruthenium(III), is more convenient. This detection method has been shown to be a sensitive and selective detection technique in high-performance liquid chromatography for the determination of amines.^{13,14} Additionally, chemiluminescence intensity increases 10-fold going from primary to secondary amines.¹⁵ We adopted this system for the reason described in the summary. A simultaneous determination of Hyp and Pro in serum was successfully performed by HPLC with chemiluminescence detection. This method is more simple and rapid

Fig. 1. Chromatogram Obtained from Deproteinized Human Serum

Peaks: A, 4-hydroxyproline (12.3 nmol/ml), B, proline (110.5 nmol/ml).

Table 1. Free 4-Hyp, Pro Content and BMD Value in Bedridden and Normal Aged Serum

	Sex	n	4-Hyp ($\mu\text{mol/l}$)	Pro ($\mu\text{mol/l}$)	BMD (g/cm^2)
Normal aged		34	10.5 ± 1.0	102 ± 10	0.284 ± 0.02 (n=27)
	F	23	11.3 ± 1.1	109 ± 14	0.256 ± 0.01 (n=21)
	M	8	8.7 ± 1.7	88.8 ± 6.0	0.381 ± 0.03 (n=6)
Bedridden aged		43	$12.5 \pm 0.5^*$	104 ± 5.4	0.257 ± 0.02 (n=32)
	F	29	$13.0 \pm 0.6^*$	103 ± 5.5	$0.209 \pm 0.0^*$ (n=24)
	M	14	$10.9 \pm 0.8^*$	105 ± 15	0.399 ± 0.04 (n=8)

Values are mean $\pm$ S.E. * $p < 0.05$ compared with each normal aged value by U-test (Mann-Whitney). F and M mean female and male, respectively.

Table 2. Free 4-Hyp, Pro Content and BMD Value within or without Fractured Bedridden Aged Serum

	Sex	n	4-Hyp ($\mu\text{mol/l}$)	Pro ($\mu\text{mol/l}$)	BMD (g/cm^2)
Without fractured		33	11.4 ± 0.5	101 ± 6.3	0.274 ± 0.03 (n=22)
	F	24	11.7 ± 0.6	98.6 ± 5.9	0.208 ± 0.02 (n=15)
Fractured		17	$14.9 \pm 1.1^*$	109 ± 10	0.219 ± 0.02 (n=10)
	F	15	$15.1 \pm 1.2^*$	111 ± 11	0.211 ± 0.02 (n=9)

Values are mean $\pm$ S.E. * $p < 0.05$ compared with each normal without fractured value by U-test (Mann-Whitney). F means female.

Fig. 2. Correlation between Free 4-Hyp (a) and Pro (b) with Normal Human Serum (Female (Open Circle) and Male (Closed Circle)) and Aging

Fig. 3. Correlation between Free 4-Hyp in Human Serum (Female (Open Cycle) and Male (Closed Cycle)) and BMD

than previously reported.^{16,17)} We believe that this method provides a useful tool for biochemical and biomedical research requiring Hyp and Pro analysis. It can be done easily just by centrifugation to cut off the high molecular compounds in serum ($MW > 10000$). Thus, it is a very convenient and useful method to determine the content of free 4-Hyp and Pro in clinical or biological inspections. In serum, Hyp exists in several types, such as free, peptide or protein. Total and free Hyp content is well correlated and free Hyp content is a reflection of total Hyp content.¹⁸⁾

Pro content in the serum of normal subjects was strictly controlled during aging (Fig. 2), whereas the free 4-Hyp content was more spread than the Pro content, and this tendency appeared much more clear past the age of 60 years. It is well known that the collagen turnover rate is suppressed by aging as increasing intracellular cross-linking deposits insoluble collagen.^{19–21)} In this experiment, it seems that the change of 4-Hyp in normal serum during aging is different between female and male, with the change in females being more gentle than in males. Thus result may reflect that bone resorption in the post middle aged female is more advanced than the same aged male. It has been reported that total Hyp in normal object serum decreases linearly during aging.¹⁰⁾ Our data is slightly different. It is not clear whether this tendency is specific for the people living in Amamioshima.

The free 4-Hyp content of bedridden aged serum shows a tendency to be elevated compared with normal aged group in Table 1. We also showed in Table 2 that the free 4-Hyp content of fractured bedridden people was higher than in those without fractures. No significant change was observed in free 4-Hyp content between bedridden aged people without fractures and normal aged people. In many cases, fractures cause aged people to become bedridden. These results showed that the free 4-Hyp content in bedridden aged people without fractures was not different from normal aged people, but with a fracture, bone resorption was further enhanced. It is well known that low BMD makes bone easy to fracture and leads to osteoporosis. The relationship between BMD value

and 4-Hyp content in serum is shown in Fig. 3. No tendency was observed in which low BMD value caused high 4-Hyp content in this study. Moreover, no significant change was observed either between less than 0.250 BMD people, which was used as a clinical basis for osteoporosis, and other people in terms of free 4-Hyp content. It seems that even in osteoporosis patients, free 4-Hyp content was not elevated dramatically.

Conclusively, free 4-Hyp in bedridden aged people was elevated from that of normal aged people. Fractures were shown to have caused this change; a possible reason is that due to the fracture, bedridden atrophy will occur and bone resorption is thus enhanced. These results also show that the free 4-Hyp content in serum is useful to clinically or biologically inspect bone resorption, especially in aged people.

Acknowledgments We would like to thank to the Yamato-son Government, Kagoshima, for donating a of serum samples; Prof. Makoto Suzuki at the University of Ryukyu for a donation good suggestion about serum Hyp; and President Hideo Mizukawa at PS Co., Ltd. for the scholarship for Yanmei Li. We likewise thank Mr. C. Alex DePaula at the University of Medicine and Density of New Jersey/Rutgers University in Biomedical Engineering for revising our manuscript, especially in English.

REFERENCES

- 1) Motegi K., Kano M., Sugano M., Yamazato F., Handa N., Namiki O., *Rinshokensa Kiki Shiyaku*, **14**(1), 7–12 (1991).
- 2) Smith R., *Clin. Sci.*, **59**(4), 215–223 (1980).
- 3) Gasser A. B., Depierre D., Courvoisier B., *Br. J. Cancer*, **39**(3), 280–283 (1979).
- 4) Okazaki I., Matsuyama S., Suzuki F., Maruyama K., Maruta A., Kubochi K., Yoshino K., Kobayashi Y., Abe O., Tsuchiya M., *J. Lab. Clin. Med.*, **120**(6), 908–920 (1992).
- 5) Bos A. J. G., Yuan P., Maruta A., Matsuyama S., Arai M., Okazaki I., *Nippon Eiseigaku Zasshi*, **49**(4), 797–806 (1994).
- 6) Borel J. P., *Ann. Biol. Clin. Paris*, **40**(5), 551–560 (1982).
- 7) Kontturi M. J., Sotaniemi E. A., Larmi T. K., *Scand. J. Urol. Nephrol.*, **8**(2), 91–95 (1974).
- 8) Powles T. J., Rosset G., Leese C. L., Bondy P. K., *Cancer*, **38**(6), 2564–2566 (1976).
- 9) Kelleher P. C., Smith C. J., *Clin. Lab. Med.*, **2**(3), 519–542 (1982).
- 10) Koyama K., Sato T., Omichi N., Miyamoto T., Mimura K., Maeda K., *Ann. Physiol. Anthropol.*, **12**(4), 243–249 (1993).
- 11) Uchikura K., Kirisawa M., *Anal. Sci.*, **7**(5), 803–804 (1991).
- 12) Uchikura K., Kirisawa M., *Anal. Sci.*, **7**(6), 971–973 (1991).
- 13) Uchikura K., Kirisawa M., Sugii A., *Anal. Sci.*, **9**(1), 121–123 (1993).
- 14) Brune S. N., Bobbitt D. R., *Anal. Chem.*, **64**(2), 166–170 (1992).
- 15) Lee W.-Y., Nieman T. A., *J. Chromatogr.*, **659**(1), 111–118 (1994).
- 16) Inoue H., Date Y., Kohashi K., Yoshitomi H., Tsuruta Y., *Biol. Pharm. Bull.*, **19**(2), 163–166 (1996).
- 17) Kataoka H., Nagao K., Makita M., *Biomed. Chromatogr.*, **7**(6), 296–300 (1993).
- 18) Uji Y., Karmen A., Okabe H., Hata K., Miura M., Ozaki K., Minamizaki M., Shibata T., Inayama S., *J. Clin. Lab. Anal.*, **8**(5), 267–272 (1994).
- 19) Geary S., Florini J. R., *J. Gerontol.*, **27**(3), 325–332 (1972).
- 20) Roberts D., Marks R., *J. Invest. Dermatol.*, **74**(1), 13–16 (1980).
- 21) Miller E. J., Rhodes R. K., “Methods in Enzymology,” ed. by Cunningham L. W., Freder D. W., Academic Press, New York, 1982, pp. 33–64.