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Original Article

Carnosine concentration in the muscle of thoroughbred horses and its implications in exercise performance

Miwako MORI[1, 2], Dai MIZUNO[3], Keiko KONOHA-MIZUNO[1], Yutaka SADAKANE[4] and Masahiro KAWAHARA[1]

[1] *Laboratory of Bio–Analytical Chemistry, Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Musashino University* *

[2] *Laboratory of Racing Chemistry* **

[3] *Department of Forensic Medicine, Faculty of Medicine, Yamagata University* ***

[4] *Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science* ****

Summary

Carnosine (β–alanyl histidine) plays essential roles in the exercise performance since it has various beneficial characteristics including pH buffering, anti-oxidant, anti–glycation, anti-crosslink activities. To investigate the effects of carnosine in the exercise of horses, we analyzed the amount of carnosine in the muscles of thoroughbred horse using high performance liquid chromatography (HPLC) equipped with a carbon column. We found the high concentrations of carnosine in horse muscles, but not of anserine (1-methyl carnosine). We detected neither carnosine nor anserine in other tissues including liver, kidney, jejunum, spleen and aorta, and found only a small amount of carnosine in esophagus, bladder, and stomach. We also investigated the variations in carnosine concentration among five muscles (flexor capri radius, triceps branchii, masseter, gluteus medius, sternocleidomastoid), and found that the gluteus medius exhibited the highest concentration of carnosine These results suggest the relationship between carnosine and muscle fiber types. Our developed methods are useful for the investigation of carnosine and the exercise performance.

Key words: exercise, muscle fiber, aging, quantitative analysis, dementia

Introduction

The exercise performance of thoroughbred racing horses is affected by factors such as body weight, muscle type, and nutrition[1]. Carnosine (β-alanyl histidine), a small and water-soluble dipeptide, in the muscle is a factor that contributes to the exercise performance of animals[2]. This peptide and its analogues anserine (1-methyl-carnosine) are present in the skeletal muscles and nervous tissues of many vertebrates[3]. The concentration of carnosine in the muscles is high. It is one of the most abundant small-molecule compounds in skeletal muscles, with concentrations similar to those of creatine and ATP. During high intensity exercise, muscle contractions lead to the production of lactic acid, and the resulting acidosis causes muscle contractile fatigue. Owing to the alkaline property of carnosine (pKa = 7.01), it is thought to play a significant role in intracellular buffering and maintaining the pH balance in muscle[4]. In addition to pH buffering, carnosine reportedly has many beneficial characteristics such as anti-oxidant, anti-glycation, and anti-crosslink activities[5-7]. Considering these characteristics, the concentration of muscle carnosine is thought to have a positive relationship with exercise performance[2, 7]. Indeed, high concentrations of muscle carnosine have been found in

* Address : 1–1–20 Shinmachi, Nishitokyo–shi, Tokyo 202–8585, Japan
** Address : 1731-2, Tsuruta-machi, Utsunomiya,Tochigi 320-0851, Japan
*** Address : 2–2–2 Iida–Nishi, Yamagata–shi, Yamagata 990–9585, Japan
**** Address : 3500-3 Minamitamagaki, Suzuka, Mie 513-8670, Japan
Correspondence to : Masahiro KAWAHARA, Ph.D., Department of Bio-Analytical Chemistry, Faculty of Pharmacy, Musashino University 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan
Tel & Fax : 81-42-468-8299, E-mail : makawa@musashino-u.ac.jp

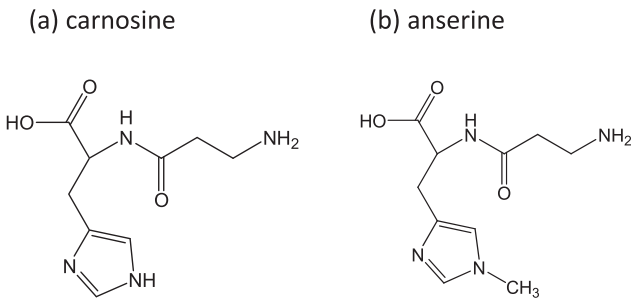

Fig. 1 The chemical structures of carnosine and anserine

several species involved in athletic competitions such as horses, dogs, and humans. Horses have 6-10 times higher muscle carnosine concentration than that in humans. Highly trained athletes have higher carnosine levels than that in untrained individuals. Furthermore, dietary supplementation of carnosine or β-alanine cause an increase in the concentration of muscle carnosine and a delay in fatigue during high-intensity exercise[8].

Therefore, quantitative analysis of muscle carnosine is crucial for the study of exercise performance in thoroughbred horses. However, as shown in Fig. 1, carnosine and anserine are highly hydrophilic, and therefore, difficult to analyze by using reversed phase high performance liquid chromatography (HPLC) equipped with a conventional octadecylsilyl (ODS) column. Here, we establish a convenient system for quantitative analysis of carnosine and anserine by using HPLC equipped with a carbon column (Hypercarb™), which contains porous graphite carbon[9]. We can measure the amount of carnosine and anserine in a sample by using a conventional UV detector. In this study, we analyzed the concentration of carnosine in thoroughbred horse muscles by using our modified HPLC system and investigated the level differences among the muscles.

Materials and Methods

Reagents

All the pharmacological reagents used were of analytical grade. Anserine, and carnosine were purchased from Sigma Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Wako Pure Chemical Industries (Osaka, Japan), and acetonitrile (CH_3CN) was purchased from Kanto Chemical (Tokyo, Japan).

Animals

We used five thoroughbred horses including four males (Horse A to D), one gelding (Horse C) and one female (Horse E), aged 4-6 years, weighting 420-505 kg. The horses were killed by administering sodium thiopental, after which muscle relaxants were injected for easy post-mortem removal of the liver, kidney, jejunum, esophagus, stomach, spleen, bladder, aorta and five muscles (flexor capri radius, triceps branchii, masseter, gluteus medius, sternocleidomastoid). Each organ was divided into three parts, and a piece of the tissue (approximately 1 cm^3) were removed from each part of these organs. These tissues were frozen in liquid nitrogen and stored at −40℃ until analysis. however, we could not obtain sternocleidomastoid muscle from horses A and B due to experimental conditions. All experimental procedures were approved by the Animal Welfare and Ethics Committee of the Equine Research Institute, Japan Racing Association in Tochigi, Japan.

Sample preparation

We placed approximately 50 mg of frozen tissue from three different part of each organ was in a Falcon tube (Thermo Fisher Scientific Inc.), added 1 mL of water, and homogenized the sample for 1 min. The samples were heated at 95℃ for 30 min to reduce the viscosity of the extracts and remove proteins, and then centrifuged at 20,000 × g for 1 h. The supernatant (20 μL) was used for HPLC analysis. To determine the recovery rate, we added 100 μmol/L of carnosine into tissue samples, and processed as described above. Each experiment was performed in triplicate.

HPLC Conditions

We used a Hitachi L-7100 HPLC system (Hitachi High Technologies Corp, Tokyo, Japan) with a Hypercarb™ column (4.6 mm i.d. × 100 mm; Thermo Electron Corp., Waltham, MA, USA). Carnosine and anserine were analyzed using an isocratic elution of 7% acetonitrile in the presence of 0.05% trifluoroacetic acid at a flow rate of 1 mL/min, and monitored at 215 nm. Each experiment was performed in triplicate. Data are expressed as means ± standard deviation (SD).

Results and Discussion

We analyzed the concentration of carnosine and anserine in extracts of horse muscles by using HPLC equipped with a carbon column. Fig. 2 shows a chromatogram of standard carnosine and anserine. Carnosine appeared at 5.6 min and anserine appeared at 7.3 min under this experimental condition. Prior to the HPLC analysis, we heated the prepared muscle samples 95℃ for 30 min, to reduce the viscosity of the extracts and to remove proteins. We examined the recovery rate of carnosine by using this pretreatment method by

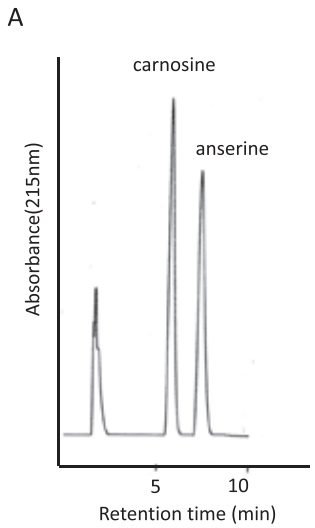

Fig. 2 Typical chromatogram for standardized carnosine
A standard solution (20 μL) of 1 mM of carnosine and anserine was analyzed using HPLC. Detection was monitored at 215 nm UV absorbance.
Eluent: 0.05%TFA: 7% CH_3CN; flow rate: 1.0 mL/min; column temperature: room temperature.

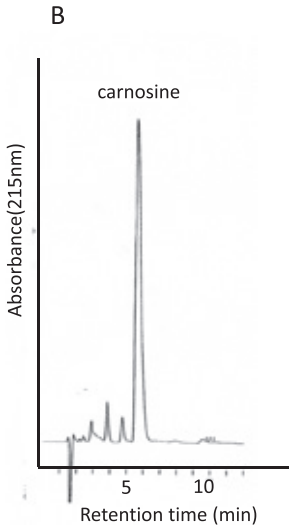

Fig. 3 Typical chromatogram for horse muscle extract
A solution (20 μL) of horse muscle extract was analyzed using HPLC. Detection was monitored at 215 nm UV absorbance. Eluent: 0.05%TFA: 7% CH_3CN; flow rate: 1.0 mL/min; column temperature: room temperature.

Table 1 Mean concentration of carnosine in selected organs

organ	Liver	Kidney	Jejunum	Esophagus	Bladder	Stomach	Spleen	Aorta
Carnosine (mg/g wet tissue)	n.d.	n.d.	n.d.	0.10 ± 0.01	0.08 ± 0.01	0.11 ± 0.03	n.d.	n.d.

Data are expressed as mean ± S.D., n = 3; n.d.: not detected.

adding a standard quantity of carnosine to the muscle extract, and found that the recovery rate ranged to 98.8 ± 6.6 % (n = 15). Thus, this simple pretreatment procedure was found to be sufficient. We used UV spectroscopy for measuring the absorbance at 215 nm, to detect of carnosine and anserine. The detection limit for our method was 0.24 μM (data not shown). Gatti *et al.* investigated the amount of carnosine and other amino acids using reversed-phase HPLC after the pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB), and detected at 360-nm absorbance using a UV spectroscope[10]. Their detection limit was 5 nmol/mL (0.022 μM). Marcia *et al.* analyzed carnosine and anserine in chicken broth using ultra performance liquid chromatography equipped with tandem mass spectrometry (MS/MS)[11]. The detection limit was 1.8 μg/L (7.9 nM). Although these method have advantages in the sensitivity compared to our method, our method has merits in the detection without derivatization using convenient and cost-effective UV spectroscopy.

An extract of horse muscle was analyzed using the same HPLC condition (Fig. 3). Although we detected carnosine in our samples, we did not detect anserine. Furthermore, we detected neither carnosine nor anserine in some organs (liver, kidney, jejunum, spleen and aorta), and found only a small amount of carnosine in the others (esophagus, bladder, and stomach) (Table 1). These results are consistent with that of previous studies[2, 3, 7, 12]. Harris *et al.* detected carnosine but not anserine in horse muscles[13]. The presence of carnosine and anserine varies among species. For example, chicken and sheep muscle contain both carnosine and anserine, but cow and pig muscle contain little or no anserine[3].

We also investigated the variations in carnosine concentration among five muscles (flexor capri radius, triceps branchii, masseter, gluteus medius, sternocleidomastoid). Table 2 shows the carnosine content of the sampled muscles from five horses. Although there are differences due to variations in sex, age, and weight, we observed a pattern in the relative concentrations of carnosine; the gluteus medius exhibited the highest concentration of carnosine among the tested muscles in most horses and the masseter showed the lowest, as shown in Fig. 4.

These findings suggest an interesting relationship between carnosine and the types of muscle fiber that influences exercise performance. There are three types of muscle fiber in horses. Type I is called slow-twitch, oxidative muscle; Type IIa is fast-twitch and oxidative-glycolytic muscle; Type IIx is fast-twitch and glycolytic

Table 2 The amount of carnosine in each horse

horse	age (years)	sex	body eight (kg)	Gluteus medius (mg/g)	Masseter (mg/g)	Flexor carpi radialis (mg/g)	Triceps brachii (mg/g)	Sternocleidomastoid (mg/g)
A	3	male	505	7.51 ± 0.28	2.45 ± 0.22	1.47 ± 0.15	2.33 ± 0.04	–
B	4	male	506	6.75 ± 0.39	2.02 ± 0.25	2.93 ± 0.12	4.76 ± 0.36	–
C	5	gelding	467	5.31 ± 0.26	1.07 ± 0.03	2.83 ± 0.29	4.18 ± 0.11	5.07 ± 0.21
D	3	male	420	4.60 ± 0.15	3.34 ± 0.18	4.60 ± 0.25	5.11 ± 0.52	5.71 ± 0.47
E	4	female	498	6.65 ± 0.34	1.04 ± 0.10	3.21 ± 0.14	4.63 ± 0.10	5.22 ± 0.44

Data are expressed as mean ± S.D., n = 3.

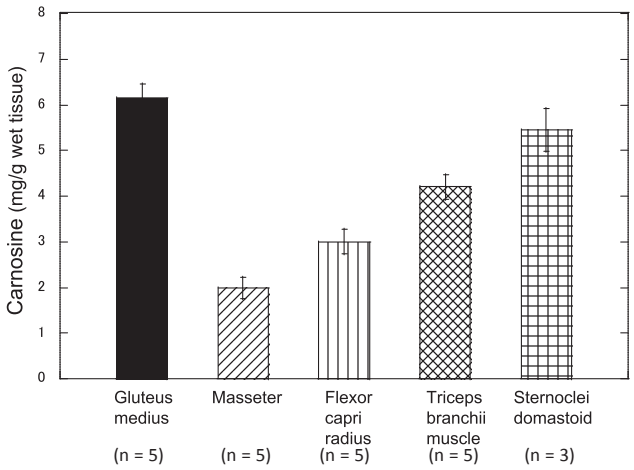

Fig. 4 The concentration of carnosine in various region of horse muscles.
Mean concentration of carnosine in each muscle of three to five horses are compared. Data are expressed as mean ± S.D., n = 3.

muscle. In general, Type IIa muscle is used for both speed and endurance and Type IIx muscle is used short-term fast running, after which the horse tires easily. Kawai *et al.* found that Type IIa and Type IIx fibers are dominant in the gluteus medius of thoroughbred horses, and that Type I was only makes up 5% of the muscle; Type IIa and Type IIx are 53% and 42% respectively[14]. Meanwhile, Type I fibers make up 78% of the masseter, and Type IIa fibers make up 76.7% of triceps branchii. The gluteus medius is mainly used in high-intensity exercise, and the masseter is used for mastication (chewing) and other slow movement.

Combining the results of previous as well as the current study, we show that the concentration of carnosine is correlated with the existence of Type IIx muscle fibers. This idea is also supported by previous studies. Sewell *et al.* reported that the concentration of carnosine in the gluteus medius of thoroughbred horses is correlated with the ratio of Type II fibers[15]. Mora *et al.* reported that in pigs, the masseter, a red oxidative muscle, showed the lowest concentration of carnosine[16]. Furthermore, Dunnett *et al.* reported that carnosine in horse plasma, which might be leakage from the muscles, changes with age, exercise experience, and muscle damage[17]. Therefore, it might be possible to estimate the exercise performance of thoroughbred horses using the concentration of carnosine in the muscle or plasma. Our modified HPLC method may provide a good tool for the measurement of carnosine concentration, and therefore, the study of exercise performance in thoroughbred horses.

It is widely known that considerable amounts of carnosine also exists in the brain, particularly in the olfactory bulbs with similar concentrations with skeletal muscles[7]. Since olfactory bulb is a 'gate' from outer circumstances into the brain, we hypothesized that carnosine in brain may act as a neuroprotector and investigated the characteristics of carnosine[18-21]. Our results and other studies suggest that carnosine and/or anserine may be effective in preventing or treating neurodegenerative diseases. Corona *et al.* reported that the dietary supplementation with carnosine attenuated memory disorder in a mouse model of Alzheimer's disease and inhibited the accumulation of β-amyloid protein in the brain of these mice[22]. The dietary supplementation with carnosine and anserine was reported to be effective for cognitive functions of elderly[23]. Therefore, our method may also be useful for the quantitative analysis of carnosine in the brain, and for the determination of physiological roles of carnosine.

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