Quantitative Assessment of Succinate Dehydrogenase Activity of Rat Lateral Pterygoid Muscle in Undecalcified Fresh-frozen Section

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Summary: The quantitative assessment of enzyme activities in situ in single muscle fibres is essential for understanding the functions of skeletal muscles. The function of the lateral pterygoid muscle (LPM) is not fully understood because it is a deeply located masticatory muscle and cannot be dissected in an intact configuration. Here we report how to measure the activities of mitochondrial succinate dehydrogenase (SDH) in single muscle fibres in situ in the LPM in sections of rat heads. Unfixed head sections were incubated on gel films containing SDH substrate and nitroblue tetrazolium. During incubation, images of the section due to deposition of the final reaction products, formazans, were captured at intervals of 10 s using a real-time image analysis system for absorbance measurements. We found that the belly of the LPM consisted of four areas with different mean activities of SDH. The lateral and upper areas of the muscle showed similarly high SDH activities. Mean activity in the lower area was the lowest, about half of those of the lateral and upper areas. These results agree with the hypothesis that the superior head of the LPM participates in more continuous contraction and is more resistant to fatigue than the inferior head.

The lateral pterygoid muscle participates in complex cooperative movements of the temporomandibular joint consisting of the mandibular fossa, head of the mandible, articular tubercle and articular disk. This muscle generally has two heads; superior and inferior1,2. Because of the unclear origin and insertion of the deeply located pterygoid muscle there is a difficulty in coordinating the muscle structure after removing it from the organism; therefore, the structure of the muscle should be studied in situ in head sections.

Measuring enzyme activities in situ in single muscle fibres provides important information about muscle functions; for example, mitochondrial succinate dehydrogenase (SDH) is a marker of oxidative (slow) fibres that are resistant to fatigue. To investigate the relation between the function and structure of the lateral pterygoid muscle, here we first quantified SDH activities in situ in single muscle fibres in the lateral pterygoid muscles in sections of rat heads and found that the muscle consisted of four areas with different mean SDH activities. The result agreed with the hypothesis of the muscle function previously reported.

Materials and Methods

Tissues

Six 10-week-old male Wister rats were purchased from Chubu Kagaku Shizai Co. Ltd., Nagoya, Japan and treated according to the guidelines for animal studies established by Aich-Gakuin University. The rats were decapitated under ether anaesthesia. Their
Heads were cut sagittally into right and left halves. The right halves were then embedded in 5% carboxymethyl cellulose gel (Finetec, Tokyo, Japan) and immediately frozen in isopentane cooled with liquid nitrogen. The frozen blocks were stored at −80°C and used for the preparation of tissue sections without fixation and decalcification.

**Tissue sections**
Frozen serial sections, 8 µm thick, of the right halves of rat heads were cut frontally at −20°C in a Leica cryostat according to the method reported previously by Kawamoto and Shimizu and Kawamoto. The frozen sections were mounted on adhesive films (Finetec) attached to coverslips (Fig. 1). These sections were used for staining with haematoxylin and eosin and for assaying succinate dehydrogenase activity in situ.

**Gel substrate films for SDH assay**
SDH was assayed in situ using a gel film technique (Fig. 1) as reported previously by Nakae and Stoward and Nakae et al. The final composition

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**Fig. 1.** On the same warm stage, a substrate gel film was mounted on a tissue section attached to adhesive film for quantification of SDH activity. From the start of quantification, a 584 nm monochromatic filter for formazan, which was the final product, was used to capture the histologic image of the lateral pterygoid muscle at 10-second intervals using the ARGUS-20 image analysis system. The background image was used for shading correction, and then the corrected image was stored.

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**Fig. 2.** Using an interference filter of 584 nm, the real-time image analysis system (IA) was used to measure absorbance (A) of seven ND filters that had previously been well established at 584 nm by a Vickers M85 scanning microdensitometer (VM). The following correlation was found between the measurement values: $A_{IA} = 0.876A_{VM} - 0.13$ ($r = 1.000, n = 7, P < 0.001$).

**Fig. 3.** Histological structure in the frontal plane of the right side of the rat head. Undecalcified fresh frozen section was used for HE staining. Arrows in Fig. 3. are (1) temporal bone, (2) lateral pterygoid muscle, (3) mandible, (4) masseter muscle, (5) zygomatic bone and (6) temporal muscle. Scale bar = 3 mm.
of substrate-containing agarose gel films for SDH was as follows: 20 mM disodium succinate (Aldrich Chemical, Milwaukee, Wisconsin, USA), 1.2 mM nitroblue tetrazolium (Sigma Chemical, St. Louis, Mo., USA), 0.26 mM phenazine methosulphate (Sigma), 10 mM NaN₃, 5 mM EDTA, 0.8% low gelling-temperature agarose (FMC BioProducts, Rockland, Me., USA), and 80 mM phosphate-buffered saline (PBS), pH 7.5. These ingredients were mixed and dissolved at 45°C in the dark. The solution was poured into the spaces of two coverslips separated by 0.6 mm-thick stainless steel spacers. It was then solidified, stored at 4°C before use and used for the assay within 6 h. Gel films in which only disodium succinate was omitted were used for measuring ‘nothing dehydrogenase’ activity without substrate.

Measurements of SDH activity in situ

Images of tissue sections being incubated with substrate gel films with or without substrate were captured and analysed using a real-time image analysis system. The system consisted of an ARGUS-20 image analyser (Hamamatsu Photonics, Hamamatsu, Japan) fitted with ARGUS-20 Plus version 1.2 Macintosh software (Hamamatsu Photonics), a microscope (Biophot; Nikon, Tokyo, Japan), a CCD camera (2400-75i; Hamamatsu Photonics), a DC power supply (PAN16-10; Kikusui Electronics, Yokohama, Japan), a thermoplate (MATS-502SF; Tokai Hit, Fujinomiya, Japan) placed on the microscope stage, an interference filter with a peak monochromatic transmission of 584 nm (Optical Coatings Japan, Tokyo, Japan), a ×20 objective (NCF Plan ELWD; Nikon, Tokyo, Japan) and a Macintosh power book G3 400 (Apple, California, USA).

First, a microscopic image of the gel substrate film sandwiched by coverslips was captured as a background image on the thermo-plate at 37°C. Second, a tissue section attached to adhesive film on a coverslip was focused on the thermo-plate. Third, the SDH reaction was started by covering the tissue section with an agarose gel film containing SDH substrate, which had been warmed at 37°C (Fig. 1). During incubation, images of the tissue section with the deposition of the final SDH reaction products, formazans, were captured at 10-s in-
tervals in real time for 4 min and saved on the hard disk after shading correction using the background image.

**Kinetic analyses**

The lateral pterygoid muscle was divided into four areas (1 upper: area 1, 2 lateral: area 2, 3 medial: area 3, and 4 lower: area 4), and in each area, 50 muscle fibers were randomly selected from the center. The absorbances of the formazans deposited in the cytoplasm (225 pixels) of single muscle fibres on the digital images were measured at each incubation time. The initial reaction velocities ($v_i$) of the SDH reaction and the control reaction (‘nothing dehydrogenase’) without substrate were determined from absorbance changes between 20 and 30 s after the start of incubation. The $v_i$ was converted into pyruvate/cm$^3$ cytoplasm/min (mmol cm$^{-3}$ min$^{-1}$) using the molar absorption coefficient of nitroblue tetrazolium formazan (16,000 l/mol/cm) at the isobestic point (585 nm)$^7$.

**Statistical analyses**

ANOVA and Fisher’s PLSD tests were applied to test statistical significances ($P < 0.01$).

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**Results**

**Calibration of the image analysis system**

Figure 2 shows the linear relation of the absorbances of the seven neutral density filters measured using our image analysis system and a Vickers M85 scanning microdensitometer.

**Histology**

Figures 3 and 4a show the frontal section of the right half of a rat head stained with haematoxylin and eosin. The belly of the lateral pterygoid muscle attached to the basal sphenoid bone was recognised. The muscle fibres were sectioned transversally. The superior and inferior heads of the muscle could not be discriminated at the belly.

**SDH activity**

The upper (1) and lateral (2) areas of the lateral pterygoid muscle showed higher SDH activities after incubation for 30 min than the medial (3) and lower (4) areas (Fig. 4b). SDH reaction products in single muscle fibres in the central lower area (4) increased with incubation time (Fig. 5). Absorbances at 584 nm of the final reaction products,

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Fig. 5. Chronological change in the digital images of formazan (A584), the final product of SDH reaction in the central lower area of lateral pterygoid muscle. Using digital images of the cytoplasm of muscle fiber (□: 225 pixels), absorbance was ascertained.
formazans, measured in single muscle fibres when incubated with a gel film with or without SDH substrate were plotted as a function of incubation time (Fig. 6). In the presence and absence of SDH substrate, the absorbances increased linearly within at least 30 s after incubation was started. Intrinsic SDH activity was determined by subtracting absorbance in the absence of the substrate from that in the presence of the substrate at each incubation time. Initial velocity was calculated from the increase of absorbances at incubation times between 20 and 30 s in intrinsic SDH reactions because the absorbance value at an incubation time of 10 s contained some errors in focussing tissue images.

Table 1 shows the mean initial velocities of the intrinsic SDH reaction in single muscle fibres in upper, lateral, medial and lower areas in the lateral pterygoid muscles in head sections of six rats. The mean initial velocity was highest in the lateral area and lowest in the lower area. The initial velocity of the lateral area was nearly twice that of the lower area. There was no significant difference in initial velocity between lateral and upper areas (Fig. 7); however, there were significant differences in other areas.

Discussion

Enzyme activities relate to the functions of muscles. The activity of mitochondrial SDH is a marker of oxidative (slow) muscle fibres that participate in

![Image](https://via.placeholder.com/150)

**Fig. 6.** Chronological changes in the absorbance of formazan (A584) using digital images of the cytoplasm of muscle fibers (225 pixels). After correcting for non-specific activity ('nothing dehydrogenase' activity), absorbance increased almost linearly for up to 30 seconds after the start.

**Fig. 7.** Data obtained from all six rats were totalled, and ANOVA and Fisher’s PLSD tests were used to analyse the mean $v_0$ of SDH activity in the four areas of the right lateral pterygoid muscle. No statistically significant difference was recognized between the upper (area 1) and lateral (area 2) areas, although significant differences ($P < 0.01$) were apparent between the other areas.

| Table 1. Mean initial velocities of the SDH reaction in four areas of rat lateral pterygoid muscles |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Mean initial velocity ($\mu$mol cm$^{-3}$ min$^{-1}$) ± SEM | Upper area 1 | Lateral area 2 | Medial area 3 | Lower area 4 |
| Rat 1 | 15.057 ± 0.317 | 14.074 ± 0.662 | 12.052 ± 0.392 | 5.450 ± 0.307 |
| Rat 2 | 14.940 ± 0.951 | 12.779 ± 0.492 | 7.443 ± 0.339 | 8.840 ± 0.347 |
| Rat 3 | 14.401 ± 0.680 | 14.903 ± 0.801 | 11.868 ± 0.544 | 9.888 ± 0.482 |
| Rat 4 | 14.405 ± 0.508 | 14.370 ± 0.656 | 10.403 ± 0.640 | 7.369 ± 0.366 |
| Rat 5 | 12.308 ± 0.667 | 14.649 ± 0.697 | 14.880 ± 0.849 | 7.630 ± 0.420 |
| Rat 6 | 11.129 ± 0.401 | 14.661 ± 0.310 | 10.637 ± 0.269 | 6.010 ± 0.514 |
| Total | 13.707 ± 0.266 | 14.239 ± 0.256 | 11.214 ± 0.257 | 7.531 ± 0.189 |
continuous SDH activity. Because of the heterogeneous SDH activities in single fibres in a muscle (e.g. Fig. 4b), they should be determined quantitatively in situ in a head section muscle. This enables us to predict the function of the muscle in situ in a living body. In the present study we found that lateral, upper, medial and lower areas in rat lateral pterygoid muscles have different mean SDH activities (Table 1 and Fig. 7). We could not discriminate the superior and inferior heads of rat lateral pterygoid muscles in the sections of muscle bellies used in the present study; however, the upper and lower halves of the muscle are considered to be the superior and inferior heads, respectively; therefore, the lateral and upper areas of rat pterygoid muscles with higher mean SDH activities (Table 1 and Fig. 7) seem to function as oxidative (slow) muscles with higher resistance against fatigue caused by continuous contraction. The lower area with lower mean SDH activity may have lower resistance against fatigue. The medial area with medium SDH activity may have an intermediate function. Our results agree with previous reports of qualitative histochemical studies of the superior and inferior heads of prerygoid muscles dissected from rats8–10. Maier8) and Katsura et al.9) suggested that the superior and inferior heads might function differently because of their different SDH activities. Oudet et al.10) reported that the superior head consisted of type I, IIA, IIB and IIC fibres but the inferior head contained only type IIA and IIB fibres.

The superior and inferior heads of pterygoid muscles showed different reciprocal contractions11–14). There is a hypothesis that the superior head, attached to the articular disk of the temporomandibular joint, contracts continuously and adjusts the relative position of the disk and the mandibular condyle for suitable mandibular movement15,16). Our results of quantifying SDH activity in situ in pterygoid muscles coincide with this hypothesis.

Our technique for quantifying enzyme activities in situ in the lateral pterygoid muscles in head sections will be a useful tool for investigating muscular dysfunction caused by damage to the movement of the temporomandibular joint.

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