Activity of Porcine Muscle Glycogen Debranching Enzyme in Relation to pH and Temperature

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[1]Abbreviations GP: glycolytic potential - LD: *longissimus dorsi* muscle - ldGDE: *longissimus dorsi* muscle glycogen debranching enzyme - M: *masseter* muscle - mGDE: *masseter* muscle glycogen debranching enzyme

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Abstract

The activity of glycogen debranching enzyme (GDE) was studied in relation to pH value and temperature in porcine *masseter* and *longissimus dorsi* muscles. A glycogen limit dextrin was used as the substrate for GDE, and the enzyme was derived from raw meat extracts. In both muscles, the pH only weakly affected on activity of GDE at the pH values found in carcasses post-slaughter. However, the activity of GDE decreased strongly (P < 0.001) when the temperature decreased from values of 39 °C and 42 °C found just after slaughter to values of 4 °C and 15 °C found during cooling. In both muscles the activity of GDE began to fall at temperatures below 39 °C and was almost zero when the temperature decreased to below 15 °C. Thus, the activity of GDE may control the rate of glycogenolysis and glycolysis, but does not block rapid glycolysis and pH decrease when the temperature is high. This may be important in PSE meat, where the pH decreases rapidly at high temperatures, but rapid cooling could limit the activity of GDE and thus glycogenolysis. As expected, GDE was more active in the light *longissimus dorsi* muscle than in the dark *masseter* muscle.

Keywords: Pig; Muscle; Glycogen debranching enzyme activity; Temperature; pH

1. Introduction

In living muscle glycogen provides local fuel storage for short-term energy consumption. After slaughter glycogen degradation to lactate causes *post-mortem* pH decline in muscles. The rate and extent of pH decrease affects several meat quality traits. The complete degradation of glycogen is

achieved by cooperation of two enzymes: glycogen phosphorylase (phosphorylase) and glycogen debranching enzyme (GDE) (Brown & Brown, 1966). Mammalian GDE is a monomeric protein containing two independent catalytic activities: a glycan transferase (EC2.4.1.25) (transferase), and amylo-1,6-glucosidase (EC3.2.1.33) (glucosidase). The two activities occur at separate sites on a single polypeptide chain (Gordon, Brown & Brown, 1972; White & Nelson, 1974; Bates, Heaton, Taylor, Kernohan & Cohen, 1975; Taylor, Cox, Kernohan & Cohen, 1975).

Phosphorylase catalyses the sequential phosphorolysis of the outer chains of the glycogen molecule until it reaches the fourth glucose unit from the branch point of the molecule (Walker & Whelan, 1960). Glycogen with four glucose units in every branch is called the limit dextrin state. The outer layer of limit dextrin has symmetric structure and it is converted back to an asymmetric structure by the transferase activity of GDE. This occurs by transferring a maltotriosyl group from the side chain to the main chain. The glucosidase hydrolyses the remaining glucosyl branch, producing free glucose. The debranched dextrin formed has long outer chains which are again susceptible to further degradation by phosphorylase (Brown & Brown, 1966; Nelson, Kolb & Larner, 1969; Nelson & Larner, 1970).

Lawrie (1955) and recently Immonen (2000) showed that *post-mortem* glycogenolysis may stop even if there is glycogen left in the muscle and speculated that GDE may play a role in this process. Yurovitzky & Milman (1975) suggested that the rate of glycogenolysis is limited by the activity of GDE. If the pH or temperature markedly affects the activity of GDE, it may influence the rate of *post-mortem* glycogenolysis and glycolysis and thus the ultimate pH of meat.

The aim of the present study was to investigate the activity of GDE in relation to pH value and temperature in porcine muscles. In addition, the pH-activity and temperature-activity profiles of

GDE and the glycolytic potential in light *longissimus dorsi* (LD) muscle and dark *masseter* (M) muscle were compared. The study focused on the combined activity of GDE only, not on the individual activities of the enzyme.

2. Materials and Methods

2.1. Animals and sampling

Muscle samples (LD and M) from 12 pigs were obtained from a commercial abattoir. The LD sample was obtained from the last rib about 35 min after stunning. The samples were diced, frozen and stored in liquid nitrogen. Freezing occurred within 1 min after sampling and the analyses were performed within two days after sampling.

The pH-activity profiles of GDE for LD and M were determined in eight animals and temperature-activity profiles in the muscles of four animals. Originally, the activity of GDE was determined at 5, 15, 25, 35, 39 and 42 °C for the temperature-activity profiles and was further determined at higher temperatures (39, 42, 50 and 60 °C) in a few additional animals. Some of the same animals were used in determining pH-activity profiles and activity at high temperatures.

2.2. Activity of the GDE

The activity of GDE was determined using the method of Nelson, Palmer & Larner (1970) with small modifications. The method follows the change in the iodine-complex spectrum of glycogen phosphorylase limit dextrin (limit dextrin), a natural substrate for GDE. In the present study the assay solution contained only 0.1 ml 1% limit dextrin and 0.020 ml 0.5 M sodium maleate (A24979, Sigma-Aldrich). Reaction was started by adding 0.08 ml meat extract. The meat extract was

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prepared daily, using 2.5 ml buffer which contained 0.05% $KHCO_3$ and 0.004 M EDTA (pH approx. 7.8 at 25 °C) per 1 g wet weight muscle. The mixture was homogenised (Ultra-Turrax T25, Janke & Kunkel, Germany) and centrifuged (Sorvall Instruments RC5C) 10 min, 10 °C and 30000 G. The supernatant was used in the measurements.

The reaction was stopped in a boiling-water bath followed by immersion in an ice bath. The reaction times were 1, 1.5 and 2.5 min for LD muscle and 1, 2.5 and 4.0 min for M muscle. Iodine reagent (2.6 ml) was added to a stopped reaction mixture and the absorbance (525 nm) recorded after 20 min. The iodine reagent was prepared according to Nelson et al. (1970) by dissolving 0.26 g of I_2 and 2.6 g KI in 10 ml water. The I_2-KI solution (1.0 ml) was added to 260 ml of saturated $CaCl_2$ solution (pH at 1 to 10 dilution 5.6 - 6.2) to produce the iodine reagent. Method blanks (zero time controls) were prepared by denaturing the meat extract protein in the boiling-water bath before adding the other reagents.

For determining the pH-activity profiles of the GDE, the pH values of the sodium maleate were pH 9.0, 6.5, 6.1, 6.0, 5.9, 5.6 or 5.1 (adjusted with HCl) and the corresponding pH of the reaction mixtures was about 7.2, 6.8, 6.4, 6.3, 6.2, 6.0 or 5.6. The maleate buffers were always heated in a water bath (90 °C) before adding to the assay solution. In this study, the assay solution was incubated at 39 °C to balance out the temperature and the reaction was started by adding the meat extract. The temperature-activity profiles of the GDE were determined similarly, but the pH of the reaction mixture was adjusted to 6.3 ± 0.05 and the reaction mixture was incubated at 4, 15, 25, 35, 39, 42, 50 or 60 °C before adding the meat extract. The temperature (39 °C) for determining the pH-activity profiles and the pH (6.3) for determining the temperature-activity profiles of GDE and the reaction times used for different muscles were defined in preliminary experiments.

The absorption spectra between 375 nm and 800 nm of the method blank and the reaction mixtures were also obtained after a 5-fold dilution of the polysaccharide-iodine solution with additional iodine reagent. All the absorbance and absorption spectra measurements were obtained with a Lambda 2 spectrometer (Perkin Elmer).

2.3. Preparation of limit dextrin

Limit dextrin is not available commercially. It was synthesised in our laboratory according to the method of Werries, Franz & Geisemeyer (1990). Commercially available rabbit muscle *phosphorylase a* (P1261, Sigma-Aldrich, St. Louis, MO, USA) was first separated from traces of GDE by passing it through a column (1.6 x 12 cm) of ω-aminobutyl agarose (A6142, Sigma-Aldrich) that was equilibrated at 4 °C with 5 mM Tris buffer (pH 7.2)/1 mM EDTA/14 mM 2-mercaptoethanol (Chen, He, Ding & Brown, 1987). Unadsorbed protein was washed off until the absorbance at 280 nm decreased to below 0.05 (Biorad Econo UV monitor EM-1, USA). The elution rate was 1 ml/min. After the first elution the column was washed with the same solvent containing 250 mM NaCl and the eluant which contained phosphorylase was collected.

The eluant was dialysed against water and lyophilised (Heto DW8-85, Hetoholten, Denmark). The activity of phosphorylase was measured spectrophotometrically (absorbance 340 nm, Perkin Elmer Lambda 2 spectrometer, Überlingen, Germany) according to Bass, Brdiczka, Eyer, Hofer & Pette (1969) following the release of glucose-1-phosphate from glycogen with L-cysteine, NaDP (N8129, Sigma-Aldrich), glucose-1,6-diphosphate, AMP, phosphoglucomutase (rabbit muscle, 79440, Fluga Chemie GmbH, Buchs, Switzerland) and glucose-6-phosphate-dehydrogenase (127671, Roche, Indianapolis, USA).

Purified, GDE-free, *phosphorylase a* was added to 350 mg glycogen (bovine liver, G0885, Sigma-Aldrich) in 5 ml 0.05 M phosphate buffer (pH 6.8). In the present study the solution also contained 0.1 mM 5'AMP (01930, Fluga). The solution was dialysed at 37 °C against 150 ml of the same buffer. The buffer was changed every 12 h and the released glucose-1-phosphate was analysed enzymatically. The digest was mixed with trichloroacetic acid (final concentration 10% v/v), dialysed against water and centrifuged for 10 min at 1000 g. The limit dextrin formed was precipitated with four volumes of ethanol, centrifuged (5 min, 1000 g), washed twice with ethanol and dried.

2.4. *Lactate and glycogen concentrations and the activity of phosphorylase*

For lactate and glycogen determinations, muscle samples were homogenised in ice-cold 0.1 M phosphate buffer (pH 7.0) with a Polytron homogeniser. The lactate concentration was determined spectrophotometrically (365 nm) using a commercial kit (Boeringer-Mannheim no. 139 084). Glycogen concentration was determined by hydrolysing the homogenate in 0.1 M HCl at 100 °C for 2 h, after which the pH was adjusted to 6.5-7.5 (Lowry & Passoneau, 1973) and glucose determined with a Roche diagnostic kit no. 1447521.

The glycolytic potential (GP) was calculated according to Monin & Sellier (1985): GP = 2([glycogen] + [glucose] + [glucose-6-phosphate]) + [lactate]. In the present study the concentrations of glucose and glucose-6-phosphate were determined simultaneously.

The activity of phosphorylase in the muscle samples was assayed as described above in connection with the preparation of phosphorylase limit dextrin.

The reproducibility of the assays was determined as follows: a large muscle sample was ground up in liquid nitrogen, diced and stored at −80 °C. This sample was analysed every time the samples were analysed. The coefficients of variation (CVs) were ±8.7%, ±10.8% and ±11.3% for the lactate and glycogen concentrations and activity of phosphorylase, respectively.

2.5. *Statistical analysis*

Statistical analysis was performed with the Statistical Analysis System version 8.02 (SAS, 1990). The REG procedure was applied when calculating the parameter estimates in the regression equation. The mixed procedure with Bonferroni adjustment was applied when calculating the least squares means of the variables in the temperature-activity profiles of GDE. The curves were fitted using SAS/insight and the curves were plotted with Microsoft Excell 97 SR-2 (XY(scatter), polynomial trendline). The pH and temperature values for maximum enzymatic activity were read from the curves whenever possible.

3. Results

3.1. *pH-activity profiles for porcine longissimus dorsi and masseter muscles GDE*

Porcine *longissimus dorsi* muscle GDE (ldGDE) breaks down its natural substrate, limit dextrin, very rapidly. The porcine *masseter* muscle GDE (mGDE) degraded limit dextrin considerably more slowly than the ldGDE; the rate of change of the absorbance per minute was slower and the overall absorbance remained low. Based on these measurements, both the ldGDE and the mGDE were active at pH values normally found in muscles *post-mortem* (Figs. 1(a) and (b)). The maximum activity of ldGDE was around pH 6.5. In the pH range studied the activity of mGDE increases slightly when the pH decreases (Fig. 1(b)).

The absorption spectra for the zero sample and for the reaction at different stages (reaction times for LD 1.0, 1.5 and 2.5 min and for M 1.0, 2.5 and 4.0 min) were scanned to ensure the progress of the reaction (data not shown). The activity of GDE causes the absorption spectra of limit dextrin to approach to that of glycogen more closely (Nelson et al., 1970). However, the spectra were not that close to the absorption spectra of glycogen, which indicates that substrate availability was not a limiting factor, i.e. the reaction had the potential to continue at maximum velocity.

3.2. Temperature-activity profiles for porcine longissimus dorsi and masseter muscles GDE

In both muscles, the activity of GDE was higher ($P < 0.001$) at those temperatures found in the carcass just after slaughter (39 °C and 42 °C) than at temperatures found during cooling (4 °C and 15 °C). In M muscle a significant ($P < 0.01$) difference was also shown in activity of GDE between temperatures of 39 °C and 25 °C.

The optimum temperature for ldGDE was 39 °C (Fig. 2(a)) and for mGDE near 50 °C (Fig. 2(b)). Activity of GDE in both muscles began to fall at temperatures below 39 °C and was almost zero when the temperature was to below 15 °C. As with the pH-activity profiles, the temperature-activity profiles also indicate that GDE was more active in light LD muscle than in dark M muscle.

3.3. Glycolytic capacity of the muscles

The glycogen and lactate concentrations, GP and activity of phosphorylase of the muscles studied are shown in Table 1. As expected, the activity of phosphorylase, lactate concentration and GP were higher and the pH value measured 35 min post-slaughter was lower in LD muscle than in M muscle. The activity of phosphorylase in LD muscle was almost 7-fold and GP 55.7 units higher than in M muscle.

4. Discussion

The pH-activity profiles were determined with maleate buffer for porcine ldGDE and mGDE over the pH range (from 5.0 to 7.4) found in muscles *post-mortem*. Various buffers affect the pH optimum of GDE (Gordon et al., 1972). In the present study, the pH only weakly affected the activity of porcine GDE. In long-lasting moderate exercise, the pH of a living muscle does not decrease, but intensive exercise may result in a sharp pH decrease, from above 7 at rest to a value as low as 6.0 during exercise (Taylor, Bore, Styles, Gadian & Radda, 1983). Hence, GDE must have the capability for being active over a large range of pH values to ensure continuous glycogenolysis and glycolysis and thus carry on energy production. Becker, Long & Fischer (1977) have found that the pH optimum for dogfish GDE was in between 5.5 and 6.3, which is close to the pH range used in the present study.

However, the pH-activity profiles of ldGDE and mGDE were different. The maximum activity of ldGDE was about pH 6.5, but a relative maximum pH value for mGDE was not found in the pH range studied. Taylor & Whelan (1968) reported that there may be two GDE isoforms in the rabbit muscle or one enzyme with a double pH optimum. They speculated that if there are two GDEs, one may have a preference for high-molecular-weight substrates. The wide pH range of the activity within muscle and the different pH-activity profiles for mGDE and ldGDE found in here may also suggest that there could be different GDE isoforms or activation mechanisms in these muscles. There is only one GDE gene in the human genome (Bao, Dawson & Chen, 1996), but recently Bao, Yang, Dawson & Chen (1997) found six GDE mRNA isoforms in human muscle. It is not yet known whether all of these isoforms can produce a functional GDE protein.

GDE was slightly more active at lower than higher pH values in the dark M muscle, possibly because in the living animal the lactate formed in type IIB muscle fibres is transported out of the fibres, either to the bloodstream or to type I muscle fibres (Halestrap & Price, 1999), resulting in a pH decrease in these fibres. Type I muscle fibres can produce energy from lactate by oxidation, thus the predominantly oxidative dark muscles (Briskey, 1964) do not need to use glycogen as an energy source when the pH is high, i.e. the activity of GDE is not critical. However, dark muscles must have the capability to break down glycogen at low pH values (pH < 6) if their oxygen stores are depleted (the low pH maximum for activity of GDE). During continuous exercise, situations may arise in which the type IIB fibres become exhausted, but the type I fibres must continue to function. Energy can in such situations still be produced anaerobically from glycogen, and thus the activity of glycogenolytic enzymes at low pH-values is critical.

In LD muscle the optimum temperature for activity of GDE was 39 °C. The activity began to rapidly to fall when the temperature decreased to below 35 °C, and the enzyme was practically inactive at temperatures below 15 °C. The other glycogen degrading enzyme, phosphorylase, is also most active at normal body temperatures (Cori, Cori & Green, 1943).

For mGDE the optimum temperature was near 50 °C. The decrease in activity of mGDE began immediately after the temperature had decreased to below body temperature (38.5±0.65 °C; Hannon, Bossone & Wade, 1990), but the decrease in activity of ldGDE did not begin until the temperature had decreased to below 35 °C. Nelson & Watts (1974) showed that the temperature optimum for rabbit muscle GDE combined activity was near 50 °C and that the activity sharply decreases when the temperature decreases to 20 °C which is consistent with the results of the present study.

We assume that the decrease in the activity of GDE caused by the temperature decrease also takes place in carcasses after slaughter. During the normal chilling procedure used in Finland, the core temperature of porcine LD muscle decreases to below 35 °C in about 1 h *post-mortem*. At that time the pH has decreased to about 6.3 and the glycogen (assumed concentration at the time of slaughter has been normal or high) is still in a state which is susceptible to degradation by phosphorylase. The temperature decrease of the muscles due to cooling of the carcass decreases the activity of GDE and limits glycogenolysis and glycolysis. Thus, the decrease in the activity of GDE may delay the rate of glycogenolysis and glycolysis *post-mortem*.

In dark M muscle the decrease in the activity of GDE and in the rate of glycogenolysis was faster than in the light LD muscle. This is supported by the findings that M muscle temperature decreases faster and that even a slight decrease in temperature significantly reduces the activity of GDE. Mélendez-Hevia et al. (1993) have estimated that at most 34.6% of glycogen molecule is directly susceptible to the degradation by phosphorylase. We estimated that this amount is enough to cause the pH to decrease from 7.0 to about 6 in dark porcine M muscle *post-mortem*. After that the activity of GDE is needed so that glycogenolysis can continue. However, the final pH in dark porcine muscles usually remains close to six: *infraspinam* 5.89±0.16 (Kylä-Puhju, Ruusunen, Kivikari & Puolanne, 2004); *masseter* 5.95±0.07 (Kylä-Puhju et al., 2004); *semispinalis* 5.90 (Lefaucheur et al., 1991), 6.23±0.33 (Przybylski, Vernin, & Monin, 1994) and *vastus intermedius* 6.23 (Bendall, 1979). Thus, the high final pH in M muscle may be due to a rapid temperature decrease in the muscle during cooling which decreases the activity of mGDE and leads to delayed glycogenolysis.

Beecher, Briskey & Hoekstra (1965) showed that *post-mortem* glycolysis is more rapid at 37 °C than at 4 °C in both light and dark portions of porcine *semitendinosus* muscle. Their results showed that the glycolytic and glycogenolytic enzymes had some activity also at low temperatures. The present study showed that the activity of GDE became very slow when the temperature decreased to below 15 °C. Beecher et al. (1965) found no significant differences between the final pH values of the light portions of *semitendinosus* muscle held at 37 °C or at 4 °C, but the final pH of the dark part of the *semitendinosus* muscle was significantly lower in muscles held at 37 °C than at 4 °C. This is also consistent with our results that show that the light LD muscle GDE had activity under 25 °C, in contrast to the dark M muscle enzyme. Thus, the decrease in activity of GDE due to temperature decrease probably stops the glycolysis earlier in the dark M muscle than in the light LD muscle.

The GP is an estimate of the glycogen content present at the moment of slaughter (Monin & Sellier, 1985) as calculated from the GP, after 35 min *post-mortem*, the glycogen concentration decreased 25.8 mmol/kg in LD muscle and only 10.5 mmol/kg in M muscle. The corresponding lactate accumulation was 51.6 ± 11.1 and 21.0 ± 4.1 mmol/kg, respectively, but the pH value was only 0.11 units lower in LD than in M muscle. This is probably the result of the higher buffering capacity in LD compared with M muscle (Kylä-Puhju et al., 2004). The rate of glycolysis was thus 2.5-fold higher in LD than in M muscle. The activity of phosphorylase was almost 7-fold higher and the activity of GDE at 42 °C 2.4-fold higher in LD than in M muscle.

The glycogen limit dextrin is the specific substrate for the debranching enzyme, and the other enzymes present in the muscles do not break it down (Nelson et al., 1970). In this study glycogen limit dextrin was used as a substrate for GDE, which was derived from raw meat extracts. Nelson et al. (1970) showed that an assay for tissue extracts utilizing a modification of the iodine-complex

spectrum for glycogen limit dextrin is specific for combined glucosidase-transferase activity. Thus, the resulting pH-activity and temperature-activity profiles for GDE were related to that part of the activity (glucosidase or transferase) that was the rate-limiting step in the debranching. Furthermore, raw meat extracts provide a natural environment for the enzymatic action. Consequently, the conditions are more similar to those actually in the cell than is the case with purified enzymes. However, probably not all glycogen occurs in limit dextrin form in living muscle. In the present study, we used glycogen limit dextrin because we focused on the role of GDE in glycogenolysis and glycolysis.

It can be concluded that GDE is more active in porcine LD muscle than in M muscle. Temperature had a greater influence than pH on activity of GDE in both muscles. GDE from both muscles is active in the pH range found in carcasses post-slaughter; however, the *post-mortem* muscle temperature decrease strongly reduces the activity of GDE and may also control glycolysis. Thus, the activity of GDE does not block rapid glycolysis and pH decrease when the temperature is high while the enzyme maintains its activity even at temperatures above 45 °C. This may be important in pale soft and exudative (PSE) meat, where the pH decreases rapidly at high temperatures, but rapid cooling could reduce GDE activity and thus glycolysis. The eventual impact of activity of GDE on PSE pork and cold shortening in beef warrants further study.

Acknowledgements

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Fig. 1. Activity of glycogen debranching enzyme from porcine *longissimus dorsi* muscle (a) and porcine *masseter* muscle (b) at different pH values.

Fig. 2. Activity of GDE from porcine *longissimus dorsi* muscle (a) and porcine *masseter* muscle (b) as a function of temperature.

Table 1. Activity of phosphorylase, glycogen and lactate concentrations, glycolytic potential and pH 35 min post-slaughter for *longissimus dorsi* and *masseter* muscles of pigs.
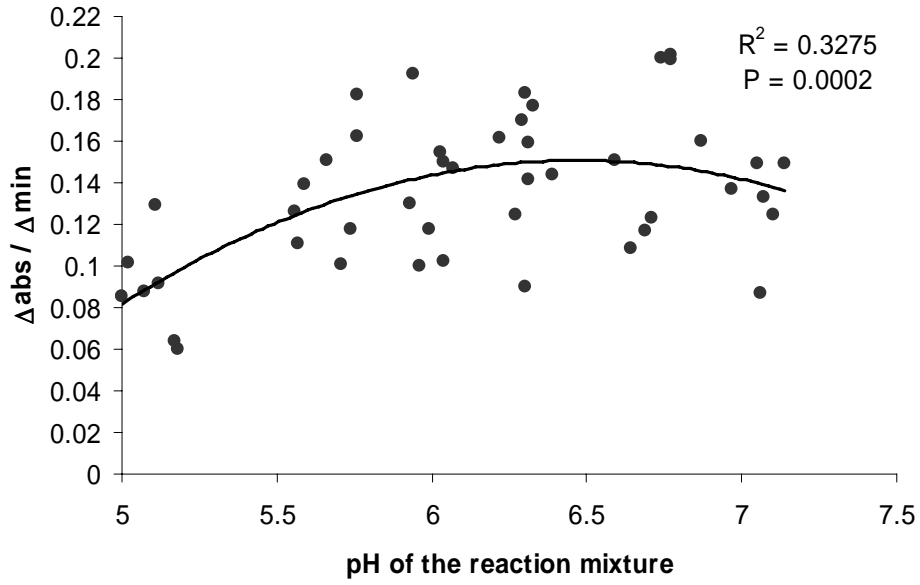
	M. longissimus dorsi	*M. masseter*	Significance
Phosphorylase, U/g muscle	14.2 ± 3.2	2.1 ± 0.7	***
Glycogen conc., mmol/kg	67.6 ± 21.8	55.1 ± 12.5	ns
Lactate conc., mmol/kg	51.6 ± 11.1	21.0 ± 4.1	***
Glycolytic potential, mmol lactate equivalent/kg muscle	186.9 ± 38.9	131.2 ± 27.6	***
pH 35 min post-slaughter	6.73 ± 0.06	6.84 ± 0.08	***

Values are mean ± STDEV (n = 12)

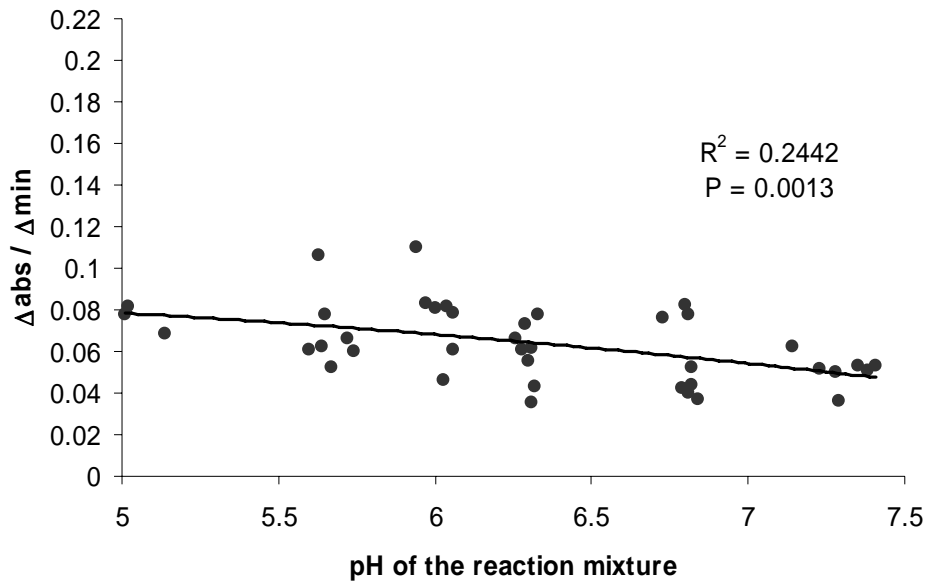*** $P < 0.001$, ns, no significant difference

Kylä-Puhju et al. (2005). Activity of Porcine... Meat Science, 69, 143-149.

Fig. 1

a)

b)

Kylä-Puhju et al. (2005). Activity of Porcine... Meat Science, 69, 143-149.

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Fig. 2

a)

b)