Optimization of muscle enzyme colorimetric tests for rapid detection of exudative pork meats.

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Running head: Enzyme assay for exudative meat detection

Abstract

The assays of muscle arginyl (RAP) and alanyl (AAP) aminopeptidases and dipeptidyl-peptidase IV (DPPIV) have been optimised using colorimetric (aminoacyl-p-nitroanilide) substrates in order to design an appropriate test in their assay in fresh pork meat. Kinetic parameters, K_m and V_{max} , were calculated for the three enzymes using pNa substrates. The rapid colorimetric analysis was developed and applied directly on the muscle extracts from 16 samples previously classified in different pork meat qualities. The exudative meat quality class (PSE) showed significant lower values for these enzyme activities than the ideal meat quality (RFN) and the dark, firm and dry (DFD) quality classes. The protocols can be easily transferred to food industry because the use of colorimetric substrates and a microplate colour reader are feasible to use in large-scale production systems.

Keywords: aminopeptidases, dipeptidyl peptidases, muscle enzymes, meat quality, proteases, exudative meats.

Introduction

The prediction of pork quality on the slaughter line is not an easy task because some of the biochemical quality properties have not enough time to be fully developed. The evaluation of pork quality should be based on relatively inexpensive and rapid measurements taken in the slaughter line where carcass identification is available, in order to choose the correct use of the product for further processing and distribution. One of the most important quality defects of pork meat is the pale, soft and exudative (PSE) meat. This PSE meat affects the quality perceived by consumers (high exudation during handling and cooking) and also by meat processors because of high losses (exudation) during processing. Another quality defect that affects consumer and processors although in low proportion, is the dark, firm and dry pork meat (DFD), characterised by its neutral pH and thus its high susceptibility to microbial development and contamination (Kauffman et al, 1992). On the other hand, the reddish pink, firm and non-exudative (RFN) meat is considered the ideal quality class meat (Kauffman et al, 1992).

During the past decades many methods have been developed to detect exudative meats, especially at early postmortem time (Kauffman et al, 1993). Several methods have been optimised at pre-rigor state such as the measurements of pH at 45 min postmortem (Somers et al, 1985) that is extensively used in slaughterhouses although presenting certain variability. Other methods consist of measurements of reflectance (Warris and Brown, 1987) and electrical conductivity (Garrido et al, 1995). At post-rigor state, the measurement of water holding capacity (Warris and Brown, 1987) and the measurements of luminosity (van Laack et al, 1994) or even the measurement of visible spectroscopy (Castro-Giráldez et al, 2010a) and dielectric properties (Castro-Giráldez et al, 2010b) were developed. From the processor point of view, it is important

to be able to predict a high water holding capacity (WHC) of meat because it is responsible for weight loss in raw, cooked and processed meats. WHC is also responsible for poor colour development in cured meat products, such as ham, and can influence meat palatability traits (Ruiz-Ramírez et al, 2005).

Proteolysis in the postmortem muscle contributes to tenderness and the development of meat flavour and therefore to the eating quality of meat (Wood et al, 1996). Firstly, the action of endopeptidases will affect the texture of the meat, the rate of tenderisation being affected by the low postmortem pH because calpain and lysosomal cathepsin activities are dependent on certain pH value (Watanabe et al, 1996). Secondly, exopeptidases act on polypeptides and proteins generating smaller peptides and free amino acids responsible for flavour changes in meat and meat products (Toldrá et al, 2009). Different factors affect the proteolytic enzyme activity detected in pork meat such as pig genotype (Hernandez et al, 2004), exercise (Lopez-Bote et al, 2008), and the presence of added salt (Armenteros et al, 2009). Therefore, the study of the proteolytic system in different pork qualities can be useful not only to find parameters that can predict technological pork quality but also for early prediction of differences in the sensory quality of pork meat. Several proteolytic enzymes have been reported as useful in the classification of different pork qualities from 2 h to 24 h postmortem (Toldrá and Flores, 2000). Among them, alanyl and arginyl aminopeptidases (AAP and RAP) and dipeptidylpeptidase IV (DPPIV) were the enzymes which showed the best statistical differences among pork quality classes. However, the use of fluorescent substrates and the need of a spectrofluorimeter to measure the enzyme activity make this process hardly applicable in a slaughter line. Therefore, the objective was to design a simple assay test for industry application using colorimetric substrates and

applied directly on the muscle extracts of fresh pork meat. Its validity was tested by comparison with pork meat samples previously classified in different qualities.

Materials and methods

Animals.

Sixteen carcasses from 6-month-old pigs (mother: Large White x Landrace, father: Large White) were selected from a local slaughterhouse and their *Longissimus dorsi* muscle were removed within 2 h postmortem. Then, loins were immediately sampled and assayed to have the enzyme and pH measurements at 2h post-mortem while the rest of the loins were kept at 4°C for the measurements of pH and colour at 24h post-mortem. The carcasses were classified, according to different measurements; pH at 2h and 24h, colour parameter L* from CIE L*, a*, b* space, measured at 24 h with a Hunter labscan Chromameter (Hunter, Reston, VA, USA) and drip loss (DL) (Warris, 1982) from two slices as described by Toldrá and Flores, (2000). The enzyme activity measurement was performed using another loin slice.

Muscle enzyme extraction.

Four grams of muscle were homogenized in 20 mL of 50 mM phosphate buffer containing 5 mM EGTA, pH 7.5, by using a polytron homogenizer (Kinematica, Luzern, Switzerland) 3 strokes, for 10 sec, at 27000 rpm while cooling in ice. The extract was centrifuged at 10000 g for 20 min at 4°C and the supernatant filtered through glass wool and used for enzyme analysis (Toldrá and Flores, 2000).

Enzyme analysis.

RAP, AAP and DPPIV were assayed using as reaction buffers those described previously by Flores et al (1993, 1996), and Blanchard et al (1993), respectively. The standard assay for each enzyme was performed by using their respective reaction buffers containing colorimetric substrates (aminoacyl-pNitroanilide derivatives: Ala-pNa for AAP, Arg-pNa for RAP and Gly-Pro-pNa for DPPIV) obtained from Sigma-Aldrich Co. (St. Louis, USA). The reaction buffer for each enzyme was as follows; RAP: 50 mM phosphate pH 6.5, 0.2 M NaCl and 0.25 mM puromycin, AAP: 100 mM phosphate pH 6.5 and 2 mM 2-mercaptoethanol, and DPPIV: 50 mM Tris-HCl pH 8.0, 1 mM DTT and 0.032 mM bestatin. The reaction mixture (300 μ L) consisted of 50 μ L of enzyme extract and 250 μ L of reaction buffer that was incubated in a multiwell plate at 37°C. Then, the released absorbance being continuously monitored at 410 nm in a microplate reader ELx800 from Biotek Inc (Winooski, VT, USA). Three replicates were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 μ mol of substrate per hour at 37°C.

Optimisation of substrate concentration.

The optimal substrate concentration was performed by calculating the kinetic parameters of muscle extract (Michaelis Menten constant (K_m) and maximum reaction velocity (V_{max})) using seven substrate concentrations within the range 0.2-1.4 mM for aminoacyl-pNa derivatives. Three replicates were measured for each experimental point. The kinetic parameters for each assay were determined by Lineweaver-Burk plots. Protein concentration in the enzyme extract was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Optimisation of muscle enzyme extract dilution.

The optimal dilution was performed by adding in the reaction mixture the enzyme extract without dilution and diluted 2, 5 and 10 times. Three replicates were measured for each experimental point.

Statistical analysis.

Linear and non-linear regressions were performed to adjust the data. Effect of the different pork meat quality classes on the enzyme activities was done by analysis of variance (ANOVA). The statistic software XLSTAT 2009.4.03 was used (Addinsoft, Barcelona, Spain). Fisher test was used to evaluate differences among treatments.

Results and discussion

Optimisation of colorimetric analysis.

Different dilutions of the enzymes in the crude extract were assayed for activity when using colorimetric substrates (Figure 1). AAP has been reported to be the aminopeptidase with the highest activity in pork muscle (Flores et al, 1996, Flores et al, 2009, 2012). Therefore the AAP activity seen in figure 1B was the maximum and the enzyme extract needs to be diluted in order to have values within a linear measurement. On the other hand, RAP (Figure 1A) and DPPIV (Figure 1C) showed linear increases in absorbance values at 410 nm during all the incubation times and in all the assayed enzyme extract dilutions. As a result, the optimal dilution of the enzyme extract and time of incubation were found to be as follows: 50% extract dilution with reaction buffer for AAP and no dilution needed for RAP and DPPIV. The selected

time for incubation was 30 min at 37°C as a linear enzyme activity for incubation up to this time was obtained through linear regressions (Figure 1).

On the other hand, different colorimetric substrate (pNA) concentrations were assayed for each enzyme in order to obtain the kinetic parameters K_m and V_{max} values in the muscle extract (Figure 2). The K_m value obtained using colour substrates (pNa) and by Lineweaver-Burk plots (Table 1), were higher than those reported for fluorimetric derivatives (Flores et al, 1993, 1996, Blanchard et al, 1993, AMC derivatives: AAP K_m : 0.240 mM, RAP K_m : 0.188 mM and DPPIV K_m : 0.039 mM) indicating a lower affinity for these pNa substrates together with a lower signal and thus, a lower sensitivity for the colorimetric assay. Nevertheless, these values allow the selection of the optimum substrate concentration for each enzyme in order to have maximal enzyme activity rate without having substrate inhibition. Thus, the substrate concentrations recommended were 1.0 mM of Ala-pNa for AAP, 2.5 mM of Arg-pNa for RAP and 0.5 mM of Gly-Pro-pNa for DPPIV. These results allowed the optimisation of the enzyme measurements for the development of rapid colour enzyme assays.

Colorimetric analyses on fresh pork meat.

These analyses were applied directly on the muscle extracts of 16 samples previously classified in different pork meat qualities. The pork quality classification was done using the measurement of pH_{2h} , pH_{24h} , colour parameter L^* , and drip loss (Table 2). Following these criteria 7 samples were classified as exudative PSE, 7 as normal meat RFN and 2 as DFD meats.

The results of RAP, AAP and DPPIV colour activities assayed in the different pork quality classes (Figure 3) showed significant lower enzyme activities in exudative (PSE) than non-exudative meats (RFN and DFD) for the three assayed enzymes. Only

RAP was not differentiated between DFD and PSE meats. This fact confirms previous studies where the measurement of these exopeptidases was adequate to discriminate between exudative and non-exudative pork meat classes (Toldrá and Flores, 2000). Although the pNa substrates have lower affinity for the enzymes than the fluorimetric substrates, the optimised pNa enzyme assays are still sensitive enough to detect the differences between quality classes. Nevertheless, this assay should be applied to a larger number of pork samples in order to select the limit value for each enzyme activity. Recent studies have confirmed the effect of different factors on pork enzyme activity like pork genotype (Hernandez et al, 2004), pork physical activity (Lopez-Bote et al, 2008), pre-freezing of hams (Flores et al, 2009) different salting processes (Flores et al, 2012) and use of ingredients like salt (Armenteros et al, 2009) and all of them were performed using fluorimetric assays. Nevertheless, the enzyme activity detected in highest proportion in pork muscle was AAP followed by RAP and DPPIV activities.

Conclusions

The development of the optimal conditions for the colorimetric measurements of the enzymes RAP, AAP and DPPIV confirms the usefulness of this assay to distinguish among different pork qualities. However, additional validation work must be carried out in further studies due to the limited number of meat samples used. Also, there are several factors (genetics, feeding, pork physical activities, pre-freezing, etc.) that should be taken into account to detect variations in the slaughterhouse measurements. These protocols can be easily transferred to the food industry. The use of colorimetric substrates and the instrument needed, just a microplate colorimetric reader, are

cheaper than fluorescence with the possibility of being applied to large-scale production systems.

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Conflict of Interest

Mónica Flores has no conflict of interest. Fidel Toldrá has no conflict of interest. This article does not contain any studies with human or animal subjects.

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Figure Legends

Figure 1. Effect of meat extract dilution on colorimetric enzyme assay for RAP (A), AAP (B) and DPPIV (C). Meat extract: without dilution ($\circ$), 1/2 dilution (Δ), 1/5 dilution ($\square$), 1/10 dilution ($\diamond$).

Figure 2. Effect of substrate concentration (pNa: p-nitroanilide derivatives) on colorimetric enzyme assays for RAP (A), AAP (B) and DPPIV (C) using the muscle extract.

Figure 3. RAP, AAP and DPPIV enzyme activities of different pork quality classes using colorimetric substrates (pNa: p-nitroanilide derivatives). Different letters in each enzyme indicate significant differences ($p < 0.05$) among quality classes. The values represent the mean and standard deviation. PSE: pale, soft and exudative meat, DFD: dark, firm and dry meat, RFN: reddish pink, firm and non-exudative meat.

Table 1. Kinetics parameters of muscle enzymes measured in the muscle extract using colorimetric substrates.

Enzyme (substrate)	Km ¹ (mM)	Vmax ² (μmol/h.mg)
AAP (Ala-pNa)	0.681	0.0069
RAP (Arg-pNa)	2.47	0.219
DPPIV (Gly-pro-pNa)	0.209	0.028

¹ Km: Michaelis-Menten constant, ²Vmax: maximum reaction velocity

Table 2. Pork quality classification based on technological parameters ^a.

	pH _{2h}	pH _{24h}	L*	DL (%)
Pale, Soft and Exudative (PSE)	< 5.8	-	> 50	> 6
Red, firm and No-Exudative (RFN)	> 5.8	< 6.0	44-50	< 6
Dark, Firm and Dry (DFD)	> 6.0	> 6.0	< 44	< 3

pH_{2h} and pH_{24h}: pH measured at 2 h and 24 h post-mortem, L*: colour coordinate L from CIE L*, a*, b*, space and DL: Drip loss.

Figure 1

Figure 2

Figure 3