POST-MORTEM PROTEOLYSIS IN FRESH PORK: ROLE OF THE LYSOSOMAL ENZYME SYSTEM AND OF THE CALPAIN SYSTEM

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Background

It is well known that the tenderness of meat increases during storage and it is generally believed to be caused by an enzymatic degradation of muscle proteins post mortem (PM). Two enzyme systems have continually been suggested as possible candidates for the proteolytic degradation PM. Several studies have suggested the lysosomal cathepsins to be involved in the tenderization process (Dutson, 1983; Etherington et al., 1987; Mikami et al., 1987; O'Halloran et al., 1997; Ouali et al., 1987). Cathepsins are able to degrade a wide spectrum of muscle proteins and have a pH optimum close to normal ultimate pH in meat which makes lysosomal enzymes good candidates for catalysing PM proteolysis and meat tenderization. In the last decade much work has implicated a major role of the calpain system for tenderization of beef and lamb. In beef the initial at-slaughter levels of the calpain system have been found to be related to the PM tenderization process and ultimate tenderness (Shackelford et al., 1991; Whipple et al., 1990; Zamora et al., 1996) and the calpain system has also been suggested to be primarily responsible for PM protein degradation and tenderness development during refrigerated storage leaving little or no importance of the lysosomal enzyme system (Hopkins and Thompson, 2002; Koohmaraie, 1996; Taylor, 2003). In pork, sparse data exists on the relationship between the initial level of enzyme activity and PM protein degradation and tenderness. However, a recent study on pork suggests that both enzyme systems could play a role (Ertbjerg et al., 1999a).

Objectives

The intentions of the present paper are to report data related to PM proteolysis in fresh pork and discuss the mechanisms of PM proteolysis and to what extent calpains and cathepsins are involved in this.

Materials and methods

Nine litters of three female pigs and three male pigs were allocated to three diet groups. Diets composition, slaughter procedure and sampling procedure were as described in Therkildsen et al. (2004). Briefly, samples for m-calpain, μ-calpain, calpastatin, cathepsin B+L, cathepsin B, β-glucuronidase and cystatin were taken 15 min after slaughter and frozen in liquid nitrogen. Temperature decline in the carcasses were measured over 24 h PM by inserting a temperature logger 10 min PM at left side LD at last rib. Samples for myofibrillar fragmentation index (MFI) were taken day 1, day 2 and day 4 PM. Calpastatin, m-calpain, μ-calpain and MFI were determined as described (Kristensen et al., 2002). Lysosomal enzyme activity and cystatin activity were done as described in Kristensen (2003). The data were tested for normal distribution using the Shapiro-Wilk test within the UNIVARIATE procedure of SAS (SAS Inst. Inc., 1999 - 2000). Variables not satisfying the requirement of a normal distribution were transformed. The effects of diet and sex were removed from all variables using the GLM procedure of SAS. Multiple linear regression models were obtained by the REG procedure of SAS using stepwise regression. A P-value of 0.15 was used to introduce and keep variables in the models.

Results

The effects of diet, sex and the interaction have consequently been removed from all data before modeling. Multiple linear regressions models were obtained using the MFI values as dependent variables and the at-slaughter activities of μ-calpain, calpastatin, cathepsin B, cystatin and the temperature decline from 60 minutes until 180 minutes PM as independent variables (Table 1). Calpastatin correlated to m-calpain ($r = 0.37$; $P = 0.009$), β-glucuronidase and cathepsin B+L to cathepsin B ($r = 0.63, 0.68$; $P = < 0.001$ & < 0.001) and μ-calpain, cathepsin B, cystatin, and temperature decline to pH45 ($R = -0.30, 0.37, 0.33, -0.50$; $P = 0.03$,

0.01, 0.02, < 0.001). To fulfill the requirement of non-covarying independent variables m-calpain, cathepsin B+L, β-glucuronidase and pH45 were excluded from the models. Modeling MFI values resulted in highly significant models explaining almost 50 % of the variation (Table 1). Calpastatin had the highest explainable power followed by cathepsin B in the models obtained for MFI values day 1 and 2 PM. At 4 days PM cathepsin B had the highest explainable power followed by calpastatin. Cystatin activity and temperature decline were included in all three models.

Table 1. Multiple linear regression models on MFI-values 1, 2 and 4 days PM.

MFI day 1		MFI day 2		MFI day 4	
Variable	Partial R^2	Variable	Partial R^2	Variable	Partial R^2
Calpastatin	0.18	Calpastatin	0.24	Cathepsin B	0.24
Cathepsin B	0.10	Cathepsin B	0.18	Calpastatin	0.15
$T_{decline}$	0.04	Cystatin	0.04	$T_{decline}$	0.04
Cystatin	0.04	$T_{decline}$	0.03	Cystatin	0.03
µ-calpain	0.03				
Model R^2	0.46		0.48		0.46
P-value	< 0.001		< 0.001		< 0.001

Discussion

The tenderization of meat is mainly caused by proteolytic degradation of muscle proteins PM. The fragmentation of myofibrils has for long been associated with tenderness development of meat (Davey and Gilbert, 1969; Møller et al., 1973) and a method to measure it has been termed MFI (Olson and Parrish, 1977). The calpain system has been suggested as the primary catalyst of the PM tenderization process in beef (Huff-Lonergan et al., 1996; Koohmaraie et al., 1986) and has also been linked to the tenderization process in pork (Ertbjerg et al., 1999a; Kristensen et al., 2002; Kristensen et al., 2003). Likewise indicators of lysosomal proteolysis PM have been linked to tenderisation in beef (Calkins et al., 1987; Ertbjerg et al., 1999b; Shackelford et al., 1991). Ouali (1992) argued that the changes in the structure of meat PM caused by proteolysis only can be explained by a synergistic action between lysosomal enzymes and the calpain system. To test this, multiple linear regression models were developed using MFI as dependent variables and the at-slaughter activity of both the calpain system and indicators of the lysosomal enzymes as independent variables (Table 1). The models obtained at all time points clearly showed that calpastatin and cathepsin B explained more of the variation in MFI collectively than each of them did in isolation, suggesting that calpastatin and cathepsin B have a co-operative action in PM proteolysis. If the calpastatin activity and the activity of cathepsin B are taken as representatives of the calpain system and of the lysosomal system, respectively, the results presented in Table 1 strongly suggest a role for both enzyme systems in PM proteolysis of pork as proposed by (Ouali, 1992).

There has been some debate regarding the involvement of the calpain system and lysosomal system in PM proteolysis and tenderization of meat, and some researchers have argued against the importance of the lysosomal system and favored the importance of the calpain system (Hopkins and Thompson, 2002; Koohmaraie, 1996; Taylor, 2003). Koohmaraie (1996) argued that no role can be assigned to the lysosomal enzymes until it is clearly confirmed that the enzymes are released from the lysosomal system during PM storage. The lysosomal proteases are encapsulated within the lysosomal system and therefore have no direct access to the myofibrillar proteins in the living animal. However, several papers report an increased activity of free lysosomal enzymes during PM storage, indicating a release from the lysosomal system after slaughter. Dutson and Lawrie (1974) observed a two fold increase in the free activity of β-glucuconidase in beef 24 hours PM which increased to three fold after 5 days storage. Ertbjerg et al. (1999b) also observed an increase of free β-glucuconidase activity and in addition an increase of the free activity of cathepsin B+L in beef during storage for 21 days. Release of cathepsin C and β-glucuconidase was also observed in lamb PM (Dutson et al., 1980). Using electron microscopy of rabbit muscle stained with anti-cathepsin D (Kubo et al., 2002) observed a gradual diffusion of cathepsin D from the lysosomes to the myofibrils during storage for 14 days. Almost all cathepsin D were released from the lysosomes and absorbed onto the myofibrils after 14 days storage. These results signify that lysosomal enzymes are released during PM storage of meat and are therefore likely to contribute to PM proteolysis. Also a significant part of the total activity of cathepsin B and cathepsin B+L can be measured in drip (Purslow et al., 2000; Kristensen, 2003). The only way cathepsin activity can be measured in drip is via a release from the lysosomal system. An other major argument against

a role for the lysosomal enzymes in muscle proteolysis during PM storage has been that no adequate explanation is provided for lack of actin and myosin degradation PM, as cathepsins can degrade myosin and actin in vitro and neither are degraded during PM storage of meat (Hopkins and Thompson, 2002; Koohmaraie, 1996; Taylor, 2003). However, degradation of both myosin and actin has recently been reported during PM storage of meat (Berge et al., 2001; Ertbjerg et al., 1999b; Lametsch et al., 2002).
The information revealed above suggest that both the lysosomal system and the calpain system significantly contribute to PM proteolysis and tenderization of meat, which is in accordance with several other studies (Dutson, 1983; O'Halloran et al., 1997; Ouali, 1992; Ouali and Valin, 1981). Ouali (1992) studied how structural changes, biochemical changes and proteolysis were affected by tenderization of beef and rabbit. The effects of tenderization were compared to the effects of incubating purified myofibrils with either lysosomal proteases or calpains. Ouali (1992) concluded that the observed changes in beef and rabbit during tenderization only can be explained by a synergistic effect of both enzyme systems. It was further suggested that the calpain system primarily is responsible for changes occurring early PM and the lysosomal system primarily responsible for changes occurring later PM. The involvement of both enzyme systems in PM proteolysis is supported by the results presented in **Table 1**. The at-slaughter activity of a representative of the lysosomal system (cathepsin B) and of the calpain system (calpastatin) were used to model the MFI values obtained from pork 1, 2 and 4 days PM. Both enzyme systems were important contributors to the three models obtained. However, calpastatin had the highest partial correlation coefficient at 1 and 2 days PM and cathepsin B the highest at 4 days PM, which fit well into the suggested time dependency of the two enzyme systems (Ouali, 1992).

Conclusions

Taken together the results presented in this paper and previous studies suggest that both the calpain system and the lysosomal enzyme system are involved in PM protein degradation in pork, and that a co-operative mode of action is a likely mechanism.

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