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Tripolyphosphate hydrolysis by bovine fast and slow myosin subfragment 1 isoforms

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Abstract

Polyphosphates are used in the meat industry to increase the water holding capacity of meat products. Tripolyphosphate (TPP) is a commonly used polyphosphate and it is metabolized into pyrophosphate and monophosphate in meat. The enzymes responsible for its metabolism have not been fully characterized. The motor domain of myosin (subfragment 1 or S1) is a likely candidate. The objectives of this study were to determine if bovine S1 hydrolyzes TPP, to characterize the TPPase activity of the fast (*cutaneous trunci*) and slow (*masseter*) isoforms, and to determine the influence of pH on S1 TPPase activity. S1 hydrolyzed TPP and in comparison with ATP as substrate, it hydrolyzed TPP 16 – 32% more slowly. Fast S1 hydrolyzed both substrates faster compared to slow S1 and the difference between the isoforms was greater with TPP as the substrate. The V_{max} was 0.94 and 5.0 nmole Pi/mg S1 protein/min while the K_m was 0.38 and 0.90 mM TPP for slow and fast S1, respectively. Pyrophosphate was a strong inhibitor of TPPase activity with a K_i of 88 and 8.3 μ M PPI for fast and slow S1 isoforms, respectively. Both ATPase and TPPase activities were influenced by pH with the activity being higher at low pH for both fast and slow S1 isoforms. The activity at pH 5.4 was 1.5 to 4 fold higher than that at pH 7.6 for the different isoforms and substrates. These data show that myosin S1 readily hydrolyzes TPP and suggest that it is a major TPPase in meat.

Introduction

Inorganic polyphosphates are used in the meat industry to increase the water holding capacity (WHC) of pork, poultry and beef products (Bernthal, 1991; Gault, 1985; Knipe, 1985; Siegel & Schmidt, 1978, 1979). An increase in WHC improves cook yield and tenderness of the resulting cooked product (Cheng & Sun, 2008). Polyphosphate use has also been linked to a decrease in lipid oxidation (Akamittath, Brekke, & Schanus, 1990; Craig, Bowers, Wang, & Seib, 1996; Tims & Watts, 1958). Polyphosphates were initially used mainly in the poultry (Schwall, Rogers, & Corbin, 1968) and fish industry (Swartz, 1970), with recent applications in pork and current studies on beef in terms of near intact muscle cuts (McGee, Henry, Brooks, Ray, & Morgan, 2003; Vote et al., 2000). Polyphosphate use in comminuted meat products improves yield and is particularly useful for production of low salt products (Barbut, Maurer, & Lindsay, 1988).

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The mechanism of action of polyphosphates to increase WHC is multi-factoral, and some of their actions are poorly understood. One mechanism is by altering the pH of the meat. Alkaline polyphosphates increase the pH away from the isoelectric point of proteins, which increases the net charge of myofibrillar proteins thus decreasing filament packing (Cheng & Sun, 2008; Martin, Atkinson, & Merrifield, 2002; Trout & Schmidt, 1983). Another mechanism that is less understood is their action on actomyosin. Polyphosphates, or mainly pyrophosphate (PPi), acts as an ATP analogue and aids in actomyosin dissociation and myosin extraction from the thick filament (Bernthal, 1991; Hamm & Neraal, 1977a; Parsons & Knight, 1990; Shen & Swartz, 2009; Xiong, Lou, Harmon, Wang, & Moody, 2000). The dissociation of the actomyosin rigor linkage allows for salt to more effectively extract myosin from the thick filaments. This extraction also enhances the binding and gelation properties of the meat.

The main polyphosphates used in the meat industry are hexametaphosphate (HMP), tripolyphosphate (TPP), and pyrophosphate (PPi) with TPP being the most commonly used polyphosphate. The rank order of the effectiveness of these polyphosphates to improve WHC is PPi > TPP > HMP (Trout & Schmidt, 1984). Of these HMP does not likely interact directly with myosin. However, TPP interacts with myosin but likely does not dissociate actomyosin or facilitate myosin extraction (Yasui, Sakanish, Hashimoto, Fukazawa, & Takahashi, 1964). Studies using PPi found that it influences actomyosin dissociation and myosin extraction from the thick filament (Brenner, Chalovich, Greene, Eisenberg, & Schoenberg, 1986; Hamm & Neraal, 1977a; Ishiwata, Muramatsu, & Higuchi, 1985; Yasui et al., 1964). Studies on the action of TPP on crude actomyosin preparations (Yasui et al., 1964) demonstrated that TPP influences actomyosin interactions only after hydrolysis to PPi, and other studies showed that TPP is hydrolyzed in meat homogenates (Neraal & Hamm, 1977b). Recent NMR studies on polyphosphate metabolism in marinated chicken breast showed that, while PPi was completely hydrolyzed to monophosphate in 1.5 hours, it takes twice as long for TPP to be completely hydrolyzed (Li, Kerr, Toledo, & Teng, 2001). Therefore, the part of the functionality of TPP is dependent upon its metabolism to PPi and the lifetime of PPi in the muscle.

The likely reaction scheme for TPP metabolism in meat is (adapted from Sutton, 1973; Weilmeier & Regenstein, 2004):

where k_1 involves TPP hydrolysis by tripolyphosphatase (TPPase) and k_2 involves hydrolysis by pyrophosphatase. The enzymes and the kinetics involved in TPP and PPi hydrolysis in meat systems are partially understood. The pyrophosphatase activity in muscle has been characterized (Morita & Yasui, 1985; Nakamura, Yamaguchi, Morita, & Yasui, 1969) and the enzyme recently isolated and characterized from fish muscle (Goa et al., 2008). The major TPPase in meat is likely myosin (Xiong, 2005), as early studies using crude actomyosin suggested (Friess & Morales, 1955; Yasui et al., 1964). However, this point needs to be confirmed using purified myosin preparations and specifically S1. If TPP is hydrolyzed by myosin, it likely follows the same pathway as the myosin ATPase (Ferenczi, Homsher, Simmons, & Trentham, 1978):

Myosin binds TPP, forming a complex. The TPPase of myosin hydrolyzes TPP into PPi and Pi, with both products still bound to myosin. Pi is released first, then PPi dissociates. Relative to TPP, the binding constant of PPi is higher (Schaub, Watterson, Loth, & Foletta, 1983). Considering this fact, PPi is likely a competitive inhibitor for the myosin TPPase and PPi has been shown to inhibit TPP hydrolysis in meat homogenates (Neraal & Hamm, 1977a).

Because muscles have variations in their flavor characteristics and textural properties due to the proportion of muscle fiber types within the structure (Xiong, 1994), it is important to determine whether the TPPase activities also differ between muscle fiber types assuming myosin is the major TPPase. Differences in ATPase activities between muscle fiber types have been observed (Seidel, Thompson, Gergely, & Sreter, 1964), with fast muscle fiber types having a higher activity than slow. Differences in the amino acid sequence in the loop 1 region of S1 is primarily responsible for variations in ATPase rates and shortening velocity between the isoforms (Sweeney et al., 1998). Comparisons of fast and slow bovine myosin heavy chain sequences showed that the loop 1 region of fast myosin had a longer amino acid sequence than slow (Chikuni, Muroya, & Nakajima, 2004).

The purpose of this study was to determine if S1 is a TPPase and if it is likely the major TPPase in meat, and whether myosin isoform, PPi and pH have an influence on its TPPase activity.

Methods

Myosin Isolation

Bovine fast (*cutaneous trunci*) myosin was purified as described in Swartz, Greaser, & Marsh (1990). Bovine slow (*masseter*) myosin was purified as described in Swartz et al. (1990) with modifications. The *masseter* muscle was excised less than 1 hour after harvest, cooled on ice for 1 hour then the surface fat and connective tissue were removed. The muscle was ground through a 3/8 inch plate. ATP was added to the phosphate/pyrophosphate extraction buffer to give a concentration of 0.1 mM and the ground muscle was extracted for 2h with occasional stirring. The pH of the extract supernatant was adjusted to 6.8-7.0 with 5M H₃PO₄ then it was diluted with nine volumes 1 mM EDTA. The crude myosin was collected by centrifugation and washed twice with 20 mM KH₂PO₄ (pH 7.0) and 1 mM EDTA. The pellets were weighed, and suspended in an equal volume of 1 M KCl, 10 mM KH₂PO₄ (pH 7.0) and 1 mM EDTA to give a final concentration of 0.5 M KCl. ATP was added to give a final concentration of 1 mM ATP then 0.75 volumes (relative to solution volume) of 2.7 M (NH₄)₂SO₄, 0.5 M KCl, 10 mM KH₂PO₄ (pH 7.0) and 2 mM EDTA were slowly added with constant stirring. The solution was stirred for another 10-20 minutes, centrifuged at 10,000 × g for 20 minutes and the supernatant was collected by filtering through glass wool. Myosin was precipitated by adding 120g/L solid ammonium sulphate then mixing for 10-20 minutes before centrifugation at 10,000 × g for 20 minutes. The resulting pellets were dissolved in a minimal volume of 0.6 M NaCl, 10 mM NaHPO₄ (pH 7.0), 1 mM EDTA and 1 mM DTT and dialyzed 4 times against 20 volumes (relative to dissolved pellet volume) of 0.6 M NaCl, 10 mM NaHPO₄ (pH 7.0), 1 mM EDTA and 1 mM DTT. The absorbance at 280 nm was measured and protein concentration estimated using an extinction coefficient of 0.56 ml/mg. For long-term storage, an equal volume of glycerol was added plus DTT to bring the latter's final concentration to 4 mM final and the protein was stored at -20°C.

Bovine S1 Purification

Bovine fast and slow S1 were isolated and purified as described in Swartz & Moss (1992) and Weeds & Pope (1977). This procedure separates out the A1 and A2 essential light chain isoforms of rabbit S1 as well as removing other peptide fragments. For bovine S1s, there was either little (fast) or no A2 isoforms so the S1 elutes as mostly one peak during the salt gradient (data not shown). The S1 was desalted on a Sephadex G-25 column equilibrated with the “base buffer” (see below) and diluted at least 5 fold in the final assay.

SDS-PAGE analyses of fast and slow S1

SDS-PAGE of samples from the columns was used to analyze the elution profile and to assess the purity of the pooled protein peaks. Gels and samples were prepared and electrophoresed, stained and de-stained as described in Fritz, Swartz, & Greaser (1989). Comparison of the pooled protein samples of the isoforms by SDS-PAGE showed that both the slow S1 motor domain and the essential light chain had higher apparent MWs than the fast (data not shown) as shown in a previous study (Shen & Swartz, 2009).

Assay Buffers and Substrate Preparation

Because Mg-ATP and likely Mg-TPP are the true substrate complexes for the myosin, and both substrates bind Mg^{2+} in a pH-dependent manner, care was taken to formulate buffers that had a constant ionic strength and either saturating Mg^{2+} (substrate-velocity studies) or a specified Mg^{2+} -substrate and free Mg^{2+} concentration (pH-activity studies). For this, the program by Fabiato (1988) was used to determine the total concentration of salt, total substrate and Mg^{2+} to give specified Mg^{2+} -substrate concentration, free Mg^{2+} and an ionic strength of 0.20M. A “base buffer” at pCa 9.0 (10^{-9} M $[Ca^{2+}]$) was made that had a final concentration of 4 mM EGTA, 1 mM NaN_3 , 1 mM DTT. Ionic strength was adjusted to 0.20 M with NaCl for all studies. The base buffer for the assays, except the pH studies, contained 20 mM PIPES (pH 7.2) and total $MgCl_2$ was 6.62 mM. For pH-activity assays, either 10 mM MOPS (pH 6.8 to 7.6) or 10 mM MES (pH 5.4 to 6.6) was used as the buffer, total Mg^{2+} was specified to give 2 mM Mg^{2+} -substrate, and 4 mM free Mg^{2+} (see tables 1 and 2 for buffer constituents). The absolute binding constants of EGTA and ATP for magnesium were from Godt & Lindley (1982), while the constants of TPP for Mg^{2+} were from Martell & Smith (1974). The binding constants were corrected for temperature and pH to give the apparent binding constants for the assay conditions (25°C and specified pH). Solution pH was adjusted at 20°C to give the desired pH at 25°C using the buffer calculator at <<http://www.liv.ac.uk/buffers/buffercalc.html>> developed by Beynon (2006). NaCl and NaOH were used instead of KCl to prevent precipitate formation with the sodium dodecyl sulfate (SDS) used in the phosphate assay. Stock solutions of 0.20 M ATP, adjusted to pH 7.0, were prepared from disodium ATP with the concentration determined using an extinction coefficient of $15,400\text{ M}^{-1}$ at 259 nm. Stock solutions of sodium tripolyphosphate (20 mM) were prepared from the dry reagent (Fluka, St. Louis, MO) in the specific assay buffer, adjusted to the desired pH for the assay and kept on ice and used within one week of preparation.

Phosphate Assay for ATPase and TPPase Activity

The assay of White (1982), which uses SDS-EDTA to stop the reaction, was adapted for a microplate-based assay and used to measure phosphate. The reaction was started by preparing 2x stock of the enzyme and 2x stock of substrate and the reaction was started by mixing 50 μ l of substrate with 50 μ l of enzyme in a 600 μ l microfuge tube. At the specified time, the reaction was stopped by adding 35 μ l of SDS-EDTA (13.3% SDS, 0.12 M EDTA, pH 7.0). The contents of the tube were added to 265 μ l of color development reagent (0.5% Fe_2SO_4 , 0.5 % ammonium molybdate, 0.5 M H_2SO_4) in a microplate well. Color was allowed to develop for 15 minutes and read in a microplate reader at 640 nm. In-plate standards (0 – 71 nmole Pi)

were prepared in the base buffer using a stock solution of KH_2PO_4 calibrated using a Sigma phosphate standard. All assays had a blank in which SDS-EDTA was added prior to substrate to correct for Pi contamination in the substrate and solution. The final S1 concentration was 1mg/ml. Assays for comparison of ATP and TPP as a substrate used 2 mM Mg^{2+} -substrate. The linearity of the assay at 0.05 mM TPP is shown in Figure 2. For the substrate comparison and substrate velocity assays, 2 – 6 time points were used, all other assays used a single time point. For these and the assays described below, each data point represents the mean rate plus or minus the standard error of three separate assays done in quadruplicate.

PPi inhibition

To determine the sensitivity of TPPase activity to PPi inhibition, TPPase activity was measured over a range of PPi levels. Pi liberated during enzyme activity was measured as described with TPP at 0.5 mM, PPi at 0.05, 0.1, 0.2, 0.5 or 1 mM in the base solution. PPi stocks were prepared from dry PPi (Fluka, St. Louis, MO) dissolved in base buffer, adjusted to pH 7.2, stored on ice and used within 1 week. Data were corrected for their respective blanks and normalized to activity at 0 mM PPi. Note that blanks with PPi alone did not have detectable Pi after 60 min of incubation, suggesting that S1 cannot hydrolyze the inhibitor (data not shown).

pH effects on S1 activity

The influence of pH on ATP and TPP activity of fast and slow S1 was determined as was done for the assays at pH 7.2. Solutions were prepared as described above and given in tables 1 and 2. The pH levels were from 5.4 to 7.6 in 0.2 pH unit increments.

Data analysis and curve fitting

Statistical analysis for isoform and substrate effects was done by simple T-tests using GraphPad Prism (GraphPad Software, La Jolla, CA). This software was also used for non-linear, least squares regression curve fitting. The $V_{\max}$ and apparent K_m for TPP activity were determined by non-linear regression to the Michaelis-Menten equation. Values for the $V_{\max}$ and K_m represent the mean and standard error of the fit. The K_i for PPi was determined from non-linear regression to the Morrison tight binding inhibitor equation (Morrison, 1969). This equation corrects total inhibitor for bound and requires known values for the K_m and enzyme concentration giving a better estimate of the K_i when binding of the inhibitor is tight. The molecular mass of S1 was assumed as 120,000 g/mol to calculate the molar concentration. The influence of pH on enzyme activity was analyzed empirically by non-linear, least squares regression to a 3rd order polynomial.

Results

TPPase and ATPase activities of fast and slow S1

Previous studies showed that ATPase activity is 2-3 times higher in rabbit fast myosin in comparison to rabbit slow myosin (Barany, Barany, Reckard, & Volpe, 1965). However, neither a comparison of TPPase activity relative to ATPase activity nor the influence of myosin isoform on TPPase activity has been determined. Figure 1 shows that fast S1 had a higher activity in comparison to slow S1 for both ATP and TPP substrates, and ATP was hydrolyzed faster than TPP. Comparison of isoforms with ATP as the substrate shows that slow S1 had 55% of the activity of fast S1. With TPP as the substrate, slow S1 had 28% of the activity of fast S1. Comparison of substrates shows that the TPP hydrolysis rate was less than 32% of ATP. Comparing the effects of isoform and substrate shows that TPPase activity of bovine slow and fast S1 were 16% and 32%, respectively, of their ATPase activities. These data show that parallel to the ATPase activity of rabbit fast and slow myosin, the TPPase activity of fast S1 was higher than slow. The difference in the activity between the two isoforms and between

the two substrates may be due to their catalytic differences. In order to address this issue, the K_m was determined for the TPPase activity of fast and slow S1.

V_{max} and K_m of TPP hydrolysis

To determine the enzymatic properties of the slow and fast S1 TPPase activity, substrate-velocity curves for TPP hydrolysis were developed to determine the V_{max} and K_m (Figure 2). The inset shows that the assay was linear at the lowest substrate concentration tested. The intercept is not zero and this may be caused by the phosphate burst that occurs in the proposed catalytic scheme but further studies are needed to confirm this. The substrate-velocity curves were analyzed by non-linear regression to obtain the V_{max} and K_m of slow and fast S1 TPPase activity. The V_{max} of TPP hydrolysis was 0.94 ± 0.088 and 5.0 ± 0.37 nmole Pi/mg S1 protein/min for slow and fast S1, respectively. The V_{max} values are lower than those obtained from figure 1 and this may have resulted from a loss of activity of the S1, especially the slow S1 during storage in the base buffer. Slow S1 lost almost 50% of its activity within 72 hours in base buffer stored on ice (data not shown). The K_m was 0.38 ± 0.13 and 0.90 ± 0.20 mM TPP for slow and fast S1 respectively. The V_{max} and K_m of slow S1 were 19% and 42% that of fast S1, respectively.

PPi Inhibition

Past studies demonstrated that the ATPase activities of myofibrils and S1 of rabbit and frog muscles were inhibited by PPi (Sleep & Glyn, 1986). Thus, it is likely that the TPPase activities of fast and slow S1 are also inhibited by PPi. To determine this, TPPase activity was determined at different PPi concentrations. As shown in Figure 3, at 1 mM PPi, inhibition of TPP hydrolysis was more than 80% for both fast and slow S1 (81% and 98% for fast and slow S1, respectively). The K_m and enzyme concentration from the substrate-velocity curve assay were used to fit the data to the Morrison tight binding K_i equation to determine the K_i values. The K_i of fast and slow S1 were 88 ± 6.9 and 18 ± 1.8 μ M PPi, with the K_i of slow S1 being 21% that of fast S1. These data show that PPi strongly inhibits S1 TPPase activity and that slow S1 is significantly more sensitive to inhibition.

Influence of pH

Previous studies showed that the ATPase of fast rabbit crude actomyosin (Yasui et al., 1964) or S1 (Schliselfeld, 1980) increased with decreasing pH but these studies did not control for ionic species and binding constants nor did they look at isoform differences. Also, the influence of pH on bovine S1 enzyme activity has not been determined. To determine the influence of pH on bovine S1 enzyme activity, ATPase and TPPase activity were measured over a wide pH range in buffers formulated to a constant Mg^{2+} -substrate concentration. As shown in Figure 4, both S1 isoforms in both ATPase and TPPase assays had a higher activity at low pH (pH 5.4) compared to the activity at high pH (pH 7.6) agreeing with earlier studies on rabbit S1 showing that S1 activity increases as pH is reduced. At high pH (7.6) the ATPase activities of fast and slow S1 were 68% and 47% in comparison to ATPase activities at low pH (5.4), whereas the TPPase activity of fast and slow S1 at high pH were 68% and 25% in comparison to the activity at low pH. Overall, the trends were similar between the isoforms for the different substrates.

Discussion

Polyphosphate solutions are widely used in the meat industry to increase WHC and thereby increase tenderness of meat products. TPP and PPi are often the preferred polyphosphates used in meat products with both being hydrolyzed to monophosphate in meat as discussed in the Introduction. The enzymes responsible for TPP hydrolysis in meat have not been fully characterized. Early studies demonstrated that rabbit actomyosin hydrolyzed TPP (Yasui et al.,

1964) and myosin has been proposed as an enzyme responsible for TPP hydrolysis in meat (Xiong, 2005). However, no studies have carefully characterized either TPP hydrolysis by S1 or the influence of myosin isoform, PPi and pH on enzyme activity. The current studies show that bovine S1 hydrolyzes TPP, but more slowly than ATP, the TPPase of slow S1 is slower than that of fast, PPi is a strong inhibitor of TPPase activity, and that TPPase activity increases with decreasing pH.

Using isolated S1, the current studies showed that the bovine myosin motor domain can readily hydrolyze TPP. This agrees with previous studies using crude rabbit actomyosin (Friess & Morales, 1955; Yasui et al., 1964). Comparison of the relative activity using ATP or TPP as the substrate shows that the ATPase activities of both fast and slow isoforms were higher (3 – 6 fold) compared to the TPPase activities of the same protein isoforms. The differences between the ATPase and TPPase activities could result from the lack of adenosine that generates the conformational change that enhances activity and/or differences in the K_m for the different substrates. The ATPase and TPPase activities of fast S1 were higher (1.8 and 3.5 fold respectively; Fig. 2) in comparison to the slow S1 activities. These data are parallel to the ATPase activities from previous studies that looked at the activities of fast and slow myosin from the cat and rabbit (Samaha, Guth, & Albers, 1970; Sreter, Seidel, & Gergely, 1966). The results from these past studies showed a 10-fold difference compared to the current results, but this could be due to species differences and the use of Ca^{2+} -ATP instead of Mg^{2+} -ATP. With TPP as the substrate, the magnitude of the difference between the isoforms is greater, thus there is a larger influence of myosin isoform on TPP hydrolysis than ATP hydrolysis.

Part of the difference observed in the activity of S1 using TPP as a substrate compared to ATP (Fig. 2) could be due to differences in the K_m resulting in incomplete saturation of the enzyme with substrate (Friess & Morales, 1955; Schliselfeld, 1980; Yasui et al., 1964). The K_m for the ATPase of rabbit S1 is 0.43 μM (Schliselfeld, 1980). Compared to the K_m values of the TPPase for both S1 isoforms, the K_m of ATPase is less than 1% the value for TPPase, pointing out the large difference in the steady state dissociation constant of TPP compared to ATP. To obtain near maximal TPPase rates, greater than 2 mM TPP is needed compared to a much lower level for ATP. This could explain some of the differences in the relative activity of ATP as the substrate compared to TPP, but when substrate is near saturating, it does not explain the catalytic differences. Another possibility is that the structure of the S1-TPP complex is different than the ATP complex resulting in slower hydrolysis. Yasui et al. (1964) showed that TPP did not dissociate actomyosin, supporting this hypothesis. In terms of comparing the TPPase K_m between the isoforms, there is at most a 2-fold difference and this does not likely explain the activity difference between the isoforms. The differences in the ATPase rate between the isoforms is likely because of differences in the ADP release rate (Siemankowski, Wiseman, & White, 1985) that result from heterogeneity in the loop 1 region between the isoforms (Chikuni et al., 2004) and the same mechanism may be responsible for the differences in TPPase activity between the isoforms. These data on the $V_{\max}$ and K_m of bovine S1 give basic information for practical applications in terms of the rate of hydrolysis of TPP as a function of time in meat.

According to Yasui et al. (1964), TPP causes changes in the actomyosin interaction only after it is hydrolyzed to PPi. Because PPi is a reaction product of TPP hydrolysis, it was important to determine if PPi is an inhibitor of the reaction, as this is important to the overall mechanism and of practical importance to understanding the metabolism of TPP in meat. Inhibition of TPPase activity increased with increasing levels of PPi and the activity was very sensitive to PPi concentration. There are no studies in the literature on PPi inhibition of TPPase activity of S1, but Sleep & Glyn (1986) showed that at 1 mM PPi the ATPase activity of rabbit myofibrils was close to 90% the activity at 0 mM PPi. The results of the current study show that the TPPase activity in the presence of 1 mM PPi was less than 20% for both fast and slow S1. This suggests that the TPPase activity of S1 has a much higher sensitivity to inhibition by PPi compared to

its ATPase activity. Just as Perry & Grey (1956) theorized about PPi inhibition of ATPase, PPi inhibition of TPPase activity could be due to the increased amount of PPi inhibiting the hydrolysis of TPP through product inhibition. Schaub et al. (1983) reported that the binding constant (K_d) for Mg-PPi is 0.37 μM , which shows that PPi binds tightly to the enzyme. This high affinity binding constant and the measured K_i values strongly suggest that PPi is a strong competitive inhibitor of TPPase activity. The practical significance of this strong inhibition in terms of a meat system is that, in the absence of pyrophosphatase activity, addition of TPP will result in hydrolysis of TPP by myosin and a subsequent build up of PPi. This build up of PPi then inhibits the TPPase activity to slow TPP metabolism in the meat.

Bovine slow S1 was over 4 fold more sensitive to inhibition by PPi than fast S1. This is congruent with the proposed mechanism that results in differences in ATPase rate between myosin isoforms in general and the proposed kinetic scheme for TPP hydrolysis by bovine S1 as described in the Introduction. Slow S1 has a slower ADP dissociation rate than fast and this is thought to be the step in the ATPase pathway that differentiates the ATPase rate of the isoforms (Siemankowski et al., 1985). In the proposed TPPase scheme, PPi is equivalent to ADP. The measured K_i is an indirect estimate of PPi dissociation rate with smaller values being associated with slower dissociation rates. Thus, differences in the TPPase rates between fast and slow S1 may be mostly determined by the differences in PPi dissociation rate rather than other steps in the pathway. Considering the slower rate of TPP hydrolysis and greater PPi inhibition, application of TPP in meat systems may be more beneficial in slow muscles as the metabolism of TPP will be slower, allowing for the PPi to influence the acto-myosin interaction for a longer time.

Post-mortem bovine muscle can have an ultimate pH in the 5.4 to 6.5 range, and these pH levels could influence the enzyme activity of S1. Previous studies showed that the ATPase of fast rabbit crude actomyosin (Yasui et al., 1964) or isolated rabbit S1 (Schliselfeld, 1980) increased with decreasing pH. These results of the current study agree with these earlier studies and show that bovine S1 follows the same trend. These earlier studies did not control for ionic species and binding constants nor did they look at isoform differences as in the current study. Considering the affinity of TPP for Mg^{2+} as a function of pH and the weak K_m of S1 for TPP as observed in the current study, it is important to maintain a constant Mg^{2+} -TPP concentration over the entire pH range so the measured rate represents the effect of pH on the enzyme and not substrate concentration. The relative insensitivity of the TPPase to pH suggests that meat pH will have no more than a 2-fold effect on TPPase activity in the functional pH range of 5.4 to 6.4. Use of alkaline forms of TPP may be more beneficial as the increase in meat pH may delay the hydrolysis of TPP.

Previous studies showed that ATPase activities of myofibrils dramatically decreased with decreasing pH at high calcium (Bowker, Grant, Swartz, & Gerrard, 2004). However, Figure 4 shows a 4 fold change or less in the ATPase or TPPase, and the ATPase and TPPase activities were both higher at pH 5.4 than pH 7.6. This suggests that, at least for the ATPase activity, the pH effect is on other regions of myosin or other proteins in the more intact myofibril system. Further studies using more complex systems (acto-S1, and/or isolated myofibrils) are needed to determine the mechanism involved in the pH effects upon ATP and TPP hydrolysis.

If myosin is the major TPPase in meat, its activity and content should be sufficient to hydrolyze TPP within the time frame that has been observed in meat systems. The TPP turnover number for fast S1 is 0.6 mole Pi/mole S1/min based upon the V_m obtained at 25°C and an assumed S1 MW of 120,000 g/mole. The concentration of myosin in fast muscle is estimated at 185 μM (Tikunov, Sweeney, & Rome, 2001) giving a maximum hydrolysis rate of about 0.11 mM Pi/min in muscle. Thus, if TPP is added to about 10 mM (close to 0.5% wt/wt), complete hydrolysis will require at least 90 minutes at 25°C in the absence of pyrophosphate inhibition.

Studies on TPP hydrolysis in meat systems show a wide range of time for complete hydrolysis being anywhere from 15 min for ground beef (Hamm & Neraal, 1977a) and salted ground chicken (Belton, Packer, & Southon, 1987), less than 50 min. in salted chicken breast (Li et al., 2001) and up to 16 h for beef (Sutton, 1973). At the extreme case, myosin easily has the capacity to be the major TPPase in meat, while in the most rapid case, other enzymes may be involved and/or the TPPase of myosin may be activated by actin and/or other factors in the meat system. Studies with ground bovine muscle showed that the TPPase activity had a pH optimum of 5.4 (Hamm & Neraal, 1977b) and that PPi inhibited the TPPase (Neraal & Hamm, 1977a). Similar features were observed with isolated S1 in the current studies suggesting that myosin may be the major TPPase in meat systems. Detailed studies of TPP metabolism in more complex systems such as isolated myofibrils and in a meat system are needed to demonstrate that myosin is the major TPPase in meat.

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Figure 1.

Comparison of ATPase and TPPase activity of slow and fast bovine S1. The activities for slow and fast S1 ATPase were 10 and 18, while the S1 TPPase activity for slow and fast were 1.7 and 5.8 nmole Pi/mg S1/min. respectively. All treatments were significantly ($p < 0.05$) different from one another.

Figure 2.

Substrate-velocity curve of slow and fast S1 TPP hydrolysis. The V_{max} and K_m values were determined using the Michaelis-Menten equation ($Y = V_{max} * [TPP] / (K_m + [TPP])$). The inset demonstrates the linearity of the assay (R^2 value = 0.96) at 0.05 mM TPP with slow S1. The V_{max} for slow and fast S1 were 0.94 ± 0.088 and 5.0 ± 0.37 nmole Pi/mg S1/min. The K_m for slow and fast S1 were 0.38 ± 0.13 and 0.90 ± 0.20 mM TPP.

Figure 3.

Inhibition of slow and fast S1 TPPase activity by PPI. The K_i values were determined through non-linear regression to the Morrison tight binding inhibitor equation $Y = V_o * (1 - (((Et + X + (K_i * (1 + (S/K_m)))) - (((Et + X + (K_i * (1 + (S/K_m))))^2 - 4 * Et * X)^{0.5}) / (2 * Et))))$. V_o is activity at 0 μM PPI, Et is total enzyme concentration in μM, X is PPI in μM, S is TPP in μM, and K_m is in μM from Figure 2. The smooth lines represent the fit. The K_i of fast and slow S1 were 88 ± 6.9 and 18 ± 1.8 μM PPI.

Figure 4.

Influence of pH on slow and fast S1 ATPase and TPPase activity. Smooth lines represent a non-linear regression fit to a 3rd order polynomial ($Y=B_0 + B_1 \cdot X + B_2 \cdot X^2 + B_3 \cdot X^3$).

ATPase activity (a) of fast and slow S1 had ranges of 27 (pH 5.4) to 18 (pH 7.6) and 21 (pH 5.4) to 10 (pH 7.6) nmole Pi/mg S1/min. TPPase activity (b) of fast and slow S1 had ranges of 8.2 (pH 5.4) to 5.5 (pH 7.6) and 4.2 (pH 5.4) to 1.1 (pH 7.6) nmole Pi/mg S1/min.

Table 1

Composition of ATPase buffers at different pH levels. Other constituents were fixed at 10 mM buffer species, 4 mM EGTA, 1 mM NaN_3 and 1 mM DTT.

pH	[NaCl]	[MgCl ₂]	[ATP]
5.4	1.59E-01	6.01E-03	2.94E-03
5.6	1.57E-01	6.02E-03	2.74E-03
5.8	1.55E-01	6.03E-03	2.56E-03
6.0	1.52E-01	6.04E-03	2.41E-03
6.2	1.49E-01	6.07E-03	2.29E-03
6.4	1.46E-01	6.11E-03	2.21E-03
6.6	1.44E-01	6.18E-03	2.15E-03
6.8	1.57E-01	6.29E-03	2.11E-03
7.0	1.54E-01	6.46E-03	2.09E-03
7.2	1.50E-01	6.74E-03	2.07E-03
7.4	1.47E-01	7.15E-03	2.06E-03
7.6	1.44E-01	7.64E-03	2.05E-03

Table 2

Composition of TPPase buffers at different pH levels. Other constituents were fixed at 10 mM buffer species, 4 mM EGTA, 1 mM NaN₃ and 1 mM DTT.

pH	[NaCl]	[MgCl ₂]	[TPP]
5.4	1.48E-01	6.01E-03	3.42E-03
5.6	1.50E-01	6.02E-03	2.71E-03
5.8	1.49E-01	6.03E-03	2.38E-03
6.0	1.47E-01	6.04E-03	2.21E-03
6.2	1.44E-01	6.07E-03	2.12E-03
6.4	1.41E-01	6.11E-03	2.07E-03
6.6	1.39E-01	6.18E-03	2.04E-03
6.8	1.52E-01	6.29E-03	2.03E-03
7.0	1.49E-01	6.46E-03	2.02E-03
7.2	1.46E-01	6.74E-03	2.01E-03
7.4	1.42E-01	7.15E-03	2.01E-03
7.6	1.39E-01	7.64E-03	2.01E-03