^{31}P NMR STUDIES OF TRIPOLYPHOSPHATE HYDROLYSIS BY PURIFIED PYROPHOSPHATASE AND TRIPOLYPHOSPHATASE IN BOVINE *SEMITENDINOSUS* MUSCLE

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Abstract – Sodium tripolyphosphate added into meat was hydrolyzed irreversibly through the action of purified pyrophosphatase (PPase) and tripolyphosphatase (TPPase). The dynamic hydrolysis of tripolyphosphate (TPP) by the PPase and TPPase in bovine muscle was studied by phosphorus-31 NMR (^{31}P NMR) spectroscopy over 24.5 h. In the presence of TPPase, the first-order reaction of TPP hydrolysis was not complete within 24.5 h, and pyrophosphate (PP) derived from TPP accumulated progressively. In the presence of both PPase and TPPase, the PP produced was subsequently hydrolyzed to Pi by PPase, and TPP was completely hydrolyzed in less than 24.5 h. The results showed that TPP was hydrolyzed faster by TPPase and PPase together than only TPPase.

Key Words – beef, polyphosphatase, polyphosphate

I. INTRODUCTION

Tripolyphosphate (TPP) has been widely used in meat processing in many countries to enhance the quality of many muscle foods. However, it added into meat was hydrolyzed irreversibly by the endogenous pyrophosphatase (PPase) and tripolyphosphatase (TPPase). Belton *et al* [1] showed that TPP was hydrolyzed completely within 90 min after addition to fresh minced chicken and the rate of orthophosphate production in second-order reactions was affected by the rate of pyrophosphate (PP) in the first order reaction production. Several studies reported that PPase have been isolated from rabbit and fish meat and their biochemical characteristics were examined [2-4]. Yao *et al* [5] and Jin *et al* [6] have done some research on PP and TPP hydrolysis in chicken breast, and demonstrated that myosin responsible for the hydrolysis of TPP is the major TPPase and several divalent cations may affect its activity. Unfortunately until now, reports on the hydrolysis of TPP in the presence of the purified PPase and TPPase were rare. In the present study, the objective was to observe the time course of hydrolysis of TPP by PPase and TPPase purified from bovine muscle using ^{31}P NMR.

II. MATERIALS AND METHODS

The following steps were carried out at 0-4 °C. The visible fat and connective tissue were removed from fresh beef *semitendinosus*, which was obtained within 2-4 h of slaughtered 36 months Simmental cattle. The muscle was cut into cubes of 0.5×0.5×0.5 cm^3. PPase and TPPase were extracted from bovine *semitendinosus* and stored at 0 °C for following experiments.

Separation and purification of PPase

The minced muscle was dispersed in sucrose solution (0.25 M) followed by homogenization for 30 s and centrifugation for 10 min at 1,000 g. The pellets were collected and dissolved in NaCl solution (0.6 M) and centrifuged for 10 min at 10,000 g. The activity of PPase was assayed in the supernatant as a crude enzyme preparation. Ammonium sulfate was added with slow stirring to reach 50% saturation and the suspension was kept for 1 h before centrifugation for 15 min at 10,000 g to remove the precipitate. To the supernatant further ammonium sulfate was added to give 70% saturation and, after 1 h, the supernatant was removed and discarded after centrifugation for 15 min at 10,000 g. The pellets were resolved in buffer (25 mM tris-maleate, 5 mM $MgCl_2$, pH 6.8) and dialyzed overnight against the same buffer, with two

changes of the buffer. The proteins were centrifuged for 10 min at 8,500g and further purified using DEAE-52 (20 cm×1.6 cm) anion-exchange column according to the method of Gao *et al* [2]. The fractions with PPase activity were collected.

Separation and purification of TPPase

TPPase from bovine *semitendinosus* was prepared following the method described previously by Jin *et al* [6]. The purified myosin was collected.

Solution of sodium tripolyphosphate (STPP) (1 g/kg) was prepared by using distilled water. The concentration of PPase and TPPase was 0.4 g/L.

Table 1 presented 4 experimental treatment groups. The treatments were referred to as a, b, c, d. All treatments were mixed well before analysis. For a, b treatments, they were assayed at 30 min. Treatments c and d were examined at 0.5 h, 4.5 h and 24.5 h. Each treatment (0.5 ml) before measurement was immediately mixed with 0.1 ml D_2O, and then was transferred to 5 mm NMR tubes for determination. All samples were prepared in triplicate for analysis for NMR measurements.

Table 1 Samples preparation for NMR

Experiment	Treatment
a	1.5 ml buffer, 0.5 ml TPPase
b	1.5 ml buffer, 0.5 ml STPP, pH 6.13
c	1.0 ml buffer, 0.5 ml TPPase, 0.5mL STPP, pH 6.33
d	0.5 ml buffer, 0.5 ml TPPase, 0.5 ml PPase, 0.5mL STPP, pH 6.55

Buffer: 25 mmol L^{-1} Tris-maleat, 0.6 mol L^{-1} NaCl, pH 5.8

The experiments were performed on a Bruker Avance 300 MHz spectrometer operating at the frequency of 161.975 MHz, in 5 mm diameter tubes at 25 °C. 85% of sodium orthophosphate was used as a standard (0 ppm) and its integral area was 1.0. The relative amounts of Pi, PP and TPP were expressed as percentages of the level of NTP in samples and calculated as the integral area of each of the phosphoric compound peak / the total areas of the phosphoric compounds peaks.

III. RESULTS AND DISCUSSION

STPP hydrolysis in the presence of TPPase

The spectrum for STPP solution showed a series of peaks (Fig 1). Peaks at -5.67 and -5.82 ppm were assigned to atom spectrum of $STPP_{ends}$. Peaks at -17.34, -17.74 and -18.22 ppm were assigned to $STPP_{internal}$ with maximal signal at -17.74 ppm. At 0.5 h, intergral area of $STPP_{end}$ and $STPP_{internal}$ were 34.74 and 16.25, respectively, while at 4.5 h and 24.5 h, intergral area of $STPP_{end}$ were 12.43 and 2.04, in the case of $STPP_{internal}$, the result were 5.79 and 1.85.

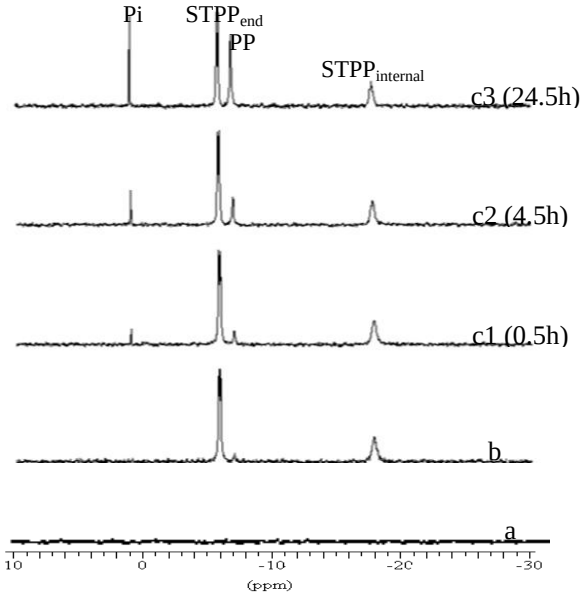

Figure 1. STPP hydrolysis in the presence of TPPase

At 0.5 h, an obvious Pi peak was observed (Fig.1 c1). The chemical shifts of $STPP_{ends}$, PP and $STPP_{internal}$ were moved to the left. Only a single peak was found in $STPP_{internal}$ and the two small peaks disappeared. STPP and PP accounted for 77.39% and 17.85% of NTP, respectively. The peak heights of Pi and PP were increased with gradually hydrolysis (Fig.1 c2 and c3). At 24.5 h, STPP and PP accounted for 32.47% and 49.97% of NTP respectively, indicating that the first stage of hydrolysis was not completed. These results were in accordance with a study in beef, showing that the stage-one hydrolysis rate (K_1) of TPP in beef was far greater than the stage-two hydrolysis rate (K_2) [7].

$$STPP \xrightarrow{K_1} PP \xrightarrow{K_2} Pi$$

STPP hydrolysis in the presence of both PPase and TPPase

After STPP hydrolysis for 0.5 h, the ^{31}P NMR spectrum for STPP showed five peaks for $STPP_{internal}$, PP, $STPP_{ends}$ (two peaks) and Pi. After treatment with the two enzymes for 4.5 h and 24.5 h, no PP peaks were observed, as the derived PP was subsequently hydrolyzed to Pi by PPase. The hydrolysis rate of STPP of the second stage (K_2) increased obviously in the presence of TPPase and PPase, leading to the peak height of $STPP_{internal}$ decreased distinctly at 4.5 h and totally hydrolysis of STPP within 24.5 h (Fig 2). Thus, STPP could be hydrolyzed more quickly in the presence of both TPPase and PPase.

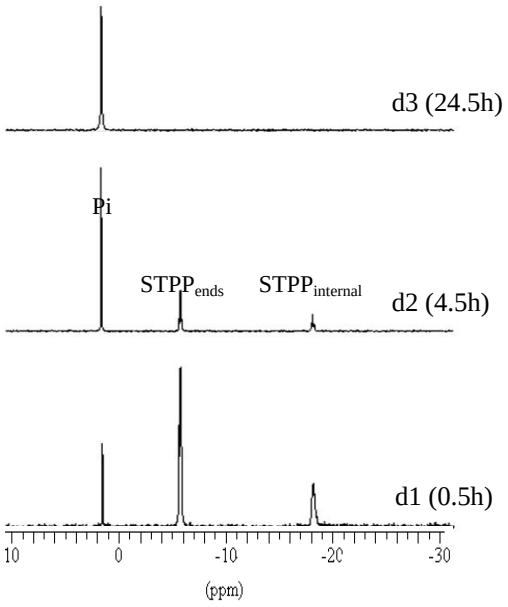

Figure 2. STPP hydrolysis in the presence of PPase and TPPase.

IV. CONCLUSION

In the system of TPPase, STPP hydrolyzed slowly and the derived PP gradually increased during STPP hydrolysis, suspecting that accumulation of the derived PP without PPase in turn led to slow hydrolysis of STPP when TPPase was applied for hydrolysis of STPP. The hydrolysis rate of STPP in the coexistence of both TPPase and PPase was faster than that in the presence of TPPase alone.

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