MEASUREMENT OF INORGANIC PYROPHOSPHATE IN SAUSAGE EMULISIONS

Jun-Ichiro MORITA*, Takao NAGAHASHI*
Atsushi TANIZAKI, and Tsutomu YASUI****

*Agricultural Experimental Farm, Faculty of Agriculture, Hokkaido University, Sapporo, 060, Japan.

**Department of Animal Science, Facutly of Agriculture, Hokkaido University, Sapporo, 060, Japan.

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Introduction

In the meat industry, inorganic polyphosphates are commonly used to improve water-holding or binding properties of cured meat products such as hams and sausages. Among polyphosphates used, only pyrophosphate (PP) has been found to be effective on the basis of its specific interaction with muscle structural proteins[1]. HASHIMOTO *et al.*[2] have pointed out that inorganic PP was hydrolyzed to orthophosphate (Pi) when it was incubated at 30°C with myosin preparation in 0.5 M KCl at pH 7.0. They determined the activity of pyrophosphatase (PPase) of the myosin preparation by measuring the increase in amount of Pi during incubation.

For the study on the effect of PP on quality of meat product, it is important to measure the remaining concentration of PP in the meat product. Foods usually contain natural phosphates beside those inorganic forms which are added during processing. To determine the amount of added phosphates, the quantity of intrinsic phosphates needs to be subtracted from the total phosphate content. However, the analysis of phosphate mixtures presents many difficulties, especially if they contain any other polyphosphates. Generally, phosphates of different chain length in foods are identified and measured by paper chromatography or thin-layer chromatography[3]. However, for quantitative measurement of each form of phosphate in foods, the contents of individual spots have to be analyzed after development of the chromatograph by wet-ashing method or by using a chromatogram-spectrophotometer and an integrator[5].

In a study on the hydrolysis of PP added to meat, application of more easier methods to determine only the PP concentration is desirable. Inorganic

PP in tissues and body fluids have been analyzed by a variety of methods. The simplest methods are based on the measurement of the chromophore produced upon reduction of the PP-molybdate complex[6~8]. However, these methods are not applicable at a Pi/PP ratio higher than 5 to 10. Since phosphorus-protein ratio (P factor) of raw lean meat is about 0.01[3], it is rather difficult to determine precisely the small amounts of PP in meat without removal of Pi by further complicated procedures[9].

Other rapid and specific methods for the determination of PP utilize enzymatic systems[4,10~13], although some of these methods need specific chemicals, which are not readily available commercially or they require extensive purification[14]. However, all chemicals required for the method of DRAKE *et al.*[4] can easily be procured from some chemical companies. In the present study, a modification of this method was applied to determine PP in meat homogenates.

Materials and Methods

Reagents: ATP sulfurylase (APSase), hexokinase, glucose-6-phosphate dehydrogenase (G-6-P DHG), adenylyl sulfate (adenosine-5′-phosphosulfate, APS), and NADP were purchased from Sigma Chemical Company. Both hexokinase and G-6-P DHG were obtained as sulfate-free, lyophilized preparations. A commercial blend of polyphosphate was obtained from Ueno Seiyaku Co., Japan. It is composed of 50% of sodium pyrophosphate (Na-PP) and 50% other sodium polyphosphates.

All other reagents were the highest grade available.

Preparation of meat samples: A rabbit was skinned and the *dorsal*, *lumbar*, and *pelvic* muscles were excised and weighed immediately after exsanguination. Porcine *longissimus dorsi* and *biceps femoris* muscles from pigs (70–90 kg, live wt) were also used one day after exsanguination. None of these pork muscles were pale, soft and exudative. The separable fat and connective tissues were removed and the muscles were ground two to three times through a chilled motor-driven grinder with a plate of 5 mm diameter holes. The ground meat (5 to 50 g) was wrapped in polyethylene films and stored in a freezer at −25°C until used for experimentation. Homogenates of different meat concentration were prepared by adding buffer solution or distilled water as needed in different experimentation in the present study. For example, 10% homogenate was prepared by adding 9 parts of buffer solution or distilled water to 1 part of minced meat and 20% homogenate was prepared by adding 8 parts of buffer solution or water to 2 parts of minced meat, so on and so forth. The mixture was homogenized twice

with an ice-cold Nihon Seiki homogenizer (HA type) each 3 min at 10,000 rpm. The pH value of the homogenate was determined with a Hitachi Horiba pH-meter (type F-5) at this stage.

Extraction of PP: The protein was precipitated by adding trichloroacetic acid (TCA) to produce a final concentration of 0.5 N in the meat homogenates before or after addition of PP. The precipitate was removed by filtration with Toyo No. 5 A filter paper. The filtrate was neutralized by adding equal voulme of 0.52 N NaOH, diluted 10- to 20-fold with distilled water, and assayed.

Assay: The method of DRAKE *et al.*[4] which is suitable for the determination of both PP and ATP was used with certain modification to determine PP. The method involves the following reactions:

$$PP+APS \xrightleftharpoons{APSase} ATP+SO_4^{2-} \quad (1)$$

$$ATP+glucose \xrightleftharpoons{Hexokinase} G\text{-}6\text{-}P+ADP \quad (2)$$

$$G\text{-}6\text{-}P+NADP \xrightleftharpoons{G\text{-}6\text{-}P\ DHG} gluconate\text{-}6\text{-}P+NADPH_2^+ \quad (3)$$

Optical density was measured with a Hitachi 100-10 spectrophotometer at 340 nm. The final volume of 1.0 ml solution contained the following components in microliters: 0.5 M imidazole-NaOH buffer (pH 6.8), 200; 2 M glucose, 100; 0.2 M $MgCl_2$, 20; 10 mM NADP, 60; 10 mM APS, 20; 100 units/ml hexokinase, 10; 100 units/ml G-6-P DHG, 10; 5 units/ml APSase, 20; diluted sample solution, 360. All stock solutions were subdivided in small test tubes and stored at −25°C. A stock of the above components except sample solution was prepared and stored at 4°C for a short time until used. A control cuvette, in which sample, APS and APSase were absent, was used as blank. After the addition of sample solution, assay mixture was incubated at 30°C for 5 min. The concentration of reduced NADP, namely PP, was calculated by using 6.22×10^3 as the molar extinction coefficient. The main differences between our method and that of DRAKE *et al.*[4] are: 1) A twofold increase in the volume of the assay mixture; 2) A decrease in the APSase concentration in the assay mixture from 1.0 to 0.1 unit; 3) A change in the buffer composition from 0.04 M triethanolamine (pH 7.6) to 0.1 M imidazole-NaOH (pH 6.8).

Preparation of experimental sausages: Sausage emulsions were prepared in accordance with commercial practice. The formulae of the sausages are shown in Table 1. Minced meat was cut with a silent cutter after

TABLE 1. The formulae of experimental sausages

Sausage	A	B	C
	(%)		
Pork lean meat	60	73	73
Pork fat	17	—	—
Ice-water	19	24	24
Polyphosphate[a]	0.3[b]	0.5[b]	0.5[c]
NaCl	2.0	2.2	2.2
Starch	2.0	—	—

[a] Per cent of the weight of lean meat.
[b] A commercial blend was used containing about 50% Na-PP and 50% other sodium polyphosphates on the weight basis.
[c] Solution containing only high grade reagent of Na-PP was used.

adding ice-cold water. During this process fat and starch were also added. Temperature of emulsion was not allowed to exceed 8°C. The pH of the emulsion was checked. Emulsion was wrapped in polyethylene film and incubated at 15°C, 50°C or 70°C. After cooking for a suitable period, the resulting experimental sausages were cooled. These samples were homogenized with TCA in the final concentration of 0.5 N, filtrated, neutralized, and assayed for residual PP concentration.

Results

Validity of the modified method for determination of inorganic pyrophosphate: The validity of the modification of the method reported by DRAKE *et al.*[4] was examined for quantitative estimation of the known amounts of PP added to meat samples. A sodium pyrophosphate (Na-PP) solution was added to 0.5 N TCA solution in the presence or absence of meat homogenate. The concentration of Na-PP was varied from 0 to 200 μM in the final assay mixture. The concentration of the meat homogenates used were from 10 to 50 per cent. After the addition of Na-PP solution, the mixtures were homogenized briefly and then the concentration of PP in the mixture was assayed as described before.

Fig. 1 shows that the optical density was linear over a range of 0.5 to 200 μM of PP. The recovery of PP added to meat homogenates was over 93% of the theoretical value in the presence or absence of 20% meat homogenate by using 6.22×10^3 as the molar extinction coefficient of reduced NADP. If the concentration of meat in homogenate was high (*e.g.* 50%), the recovery was low (90%).

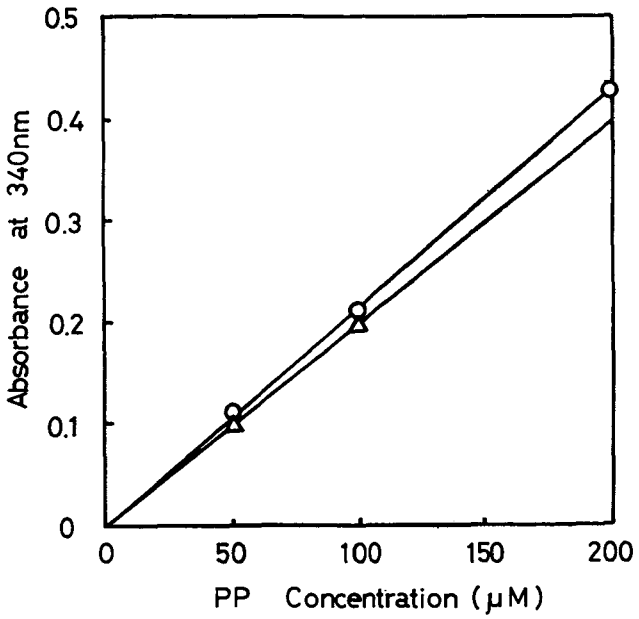

Fig. 1. Standard curve obtained with the modified enzymic method. After addition of 1.5-fold volume of 1 N TCA solution to the 10% (○) or 20% (△) porcine muscle homogenate, 0.5-fold volume of Na-PP stock solution was mixed. Neutralized solution was diluted 10-fold with water. Assay conditions have been descirbed in "Materials and Methods".

Hydrolysis of PP by meat homogenate: Na-PP was added at 0.3% and 2.2% to rabbit meat homogenate on weight basis, because more than 0.3% polyphosphate blends are used in meat industry. As shown in Fig. 2, added PP was hydrolyzed by PPase during incubationat at 25°C for 50 min. Since these plots show a simple delay curve, the rate of hydrolysis could be expressed by a reciprocal of half-life, $t_{1/2}$, from these plots. The hydrolytic activity was slightly higher in the homogenate perpared at 25 h than 1.5 h post-mortem. This suggested that the hydrolytic activity was affected by changes in post-mortem. Since pH of muscle drops post-mortem due to formation of lactic acid, pH of the homogenate was adjusted by adding buffer solutions. Fig. 3 shows that the rate of hydrolysis was influenced by pH. The rate was higher at pH 7.2 than at pH 5.6. Figs. 2 and 3 also depict that the hydrolytic activity was directly proportional to the concentration of meat in the homogenate. Frozen, long-time stored procine meat was used in similar experiments. The rate of hydrolysis depended on storage time and storage conditions. The activity of PPase in fresh meat was higher than in post-rigor meat.

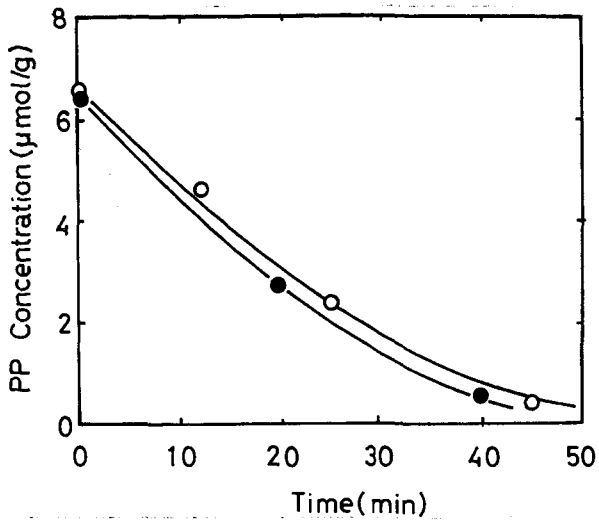

Fig. 2. Hydrolysis of PP by rabbit meat homogenate with high content of meat. Fifteen mililiters of 13.5 mM Na-PP soultion was added to 30 g of ground meat prepared from a rabbit carcass at 1.5 h (○) and 25 h (●) post-mortem. Weight ratio of Na-PP to meat was 0.003 (6.73 μmol Na-PP/g meat). After homogenization, pH of the homogenates prepared 1.5 h and 25 h post-mortem was 6.03 and 5.71, respectively. Samples were taken from the homogenates at suitable times after incubation at 25°C.

Effect of pH and temperature on the hydrolysis of PP: The pH of porcine homogenates was adjusted from 4.6 to 7.8 by adding acid or base in order to examine the effect of pH on the hydrolytic activity in more wide pH range. As shown in Fig. 4, two hydrolytic activity peaks were found: one was at about pH 5.5, and the other was at about pH 7.2. The activity at neutral pH was about 3 times higher than that in acid region.

Temperature of incubation was increased gradually from 0 to 80°C for examining the effect of temperature on the hydrolysis of PP. Fig. 5 shows temperature-dependent changes in the reaction rate. The plots of hydrolysis rate versus temperature show different profile of the curves with varying pH of homogenate. The sample with pH 7.0 showed maximum activity at 70°C, whereas samples with pH 5.6 indicated maximum activity at 60°C. Beyond these temperatures, the activity of PPases decreased possibly due to heat denaturation. These results suggest that PPases in muscle were more stable at pH 7.0 than at pH 5.6.

Hydrolysis of polyphosphates in experimental sausages: A commercial blend of polyphosphate (sausage A and B) or a high grade Na-PP

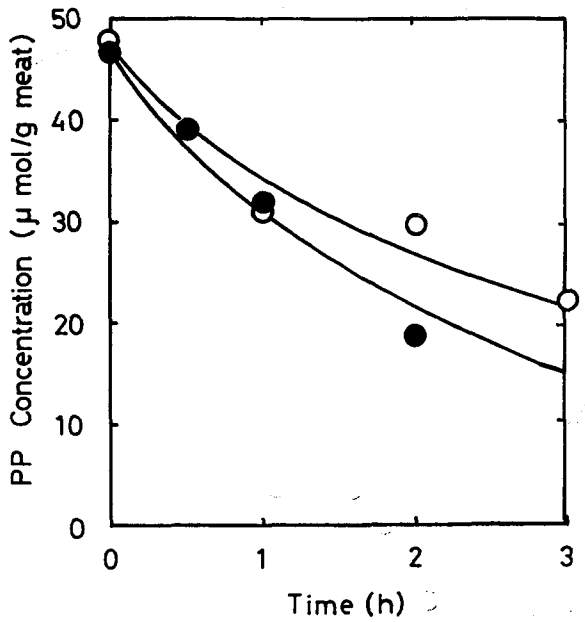

Fig. 3. Hydrolysis of PP by rabbit meat homogenate with low content of meat. Forty-five mililiters of buffer solution containing 0.1 M sodium-acetate buffer, pH 5.6 (○), or 0.1 M Tris-imidazole buffer, pH 7.2 (●), was added to 5 g of ground meat prepared at 5.5 h post-mortem. After homogenization, 0.5 mℓ of 20 mM Na-PP solution was mixed with 2 mℓ of the homogenate. Weight ratio of Na-PP to meat was 0.022 (50 μmol Na-PP/g meat). The mixture was stirred in vessel for a suitable time at 25°C, and then TCA solution was added to stop the reaction.

(sausage C) was added to experimental sausage emulsions (Table 1), and then sausage emulsions were incubated at 15°C, 50°C, or 70°C. Table 2 shows PP concentration in the sausage emulsions after incubation for a suitable time. At 15°C, PP in sausage C was largely decreased but not in sausage A or B during incubation for 120 min. On incubating at 50°C for 60 min, almost all PP in sausage C was hydroluzed, but about 70% of the initial amounts of PP were detected in sausage A and B. On the other hand, the amounts of PP detected in sausage A, B, and C were 88%, 90%, and 54%, respectively, of the initial values after incubation for 60 min at 70°C.

Discussion

One of the objectives of this study was to confirm the validity of our modification of the method of DRAKE *et al.*[4] for the determination of PP contents in meat homogenate. Since enzyme APSase is rather expensive,

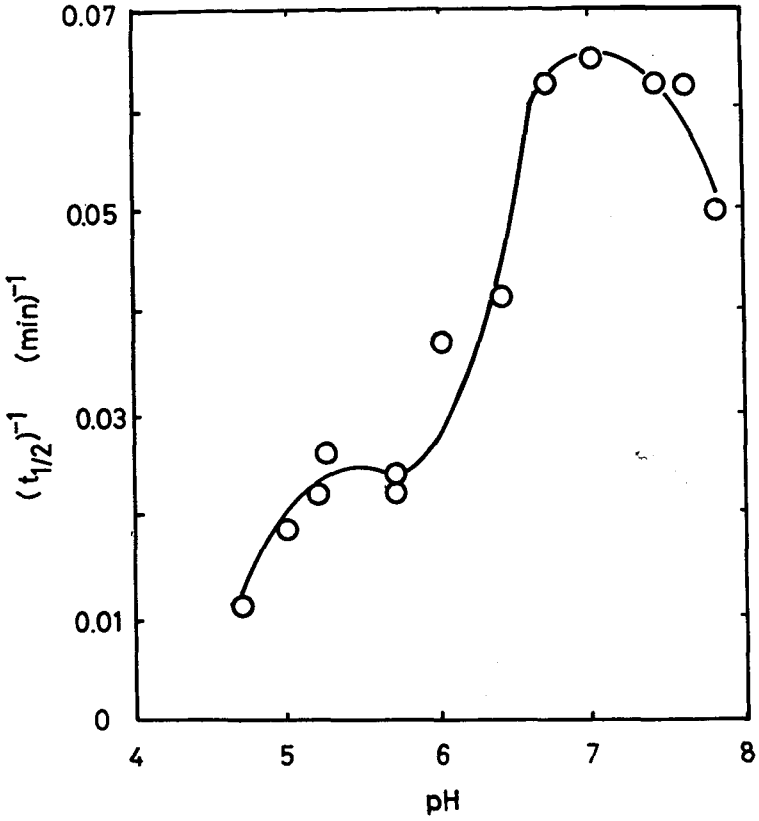

Fig. 4. Effect of pH on the hydrolysis of PP. The desired pH of porcine homogenate was adjusted by adding 10 mℓ of NaOH or HCl solution of different normalities to 15 g of ground meat to keep the volume constant. After homogenization for 1 min, 5 mℓ of 33.6 mM Na-PP solution was added. The samples were again homogenized twice, each for 3 min. Weight ratio of Na-PP to meat was 0.005 (11.2 μmol Na-PP/g meat). The pH of the homogenate was recorded. Samples were taken after suitable incubation intervals at 30°C and the contents of unhydrolyzed PP in the sample was determined.

we examined the possibility of using this enzyme at lower concentration than what DRAKE *et al.*[4] had employed without affecting the precision of the results. We found that APSase at concentration of 1/10 that used by DRAKE *et al.* gave identical results as obtained with the original method. With our modified method the added PP could be determined in the meat homogenate quantitatively (Fig. 1), and the recovery was more than 90 per cent of the theoreticaly calculated value. In the absence of meat homogenate, we had also experienced that the absorbancy of blank increased on incuba-

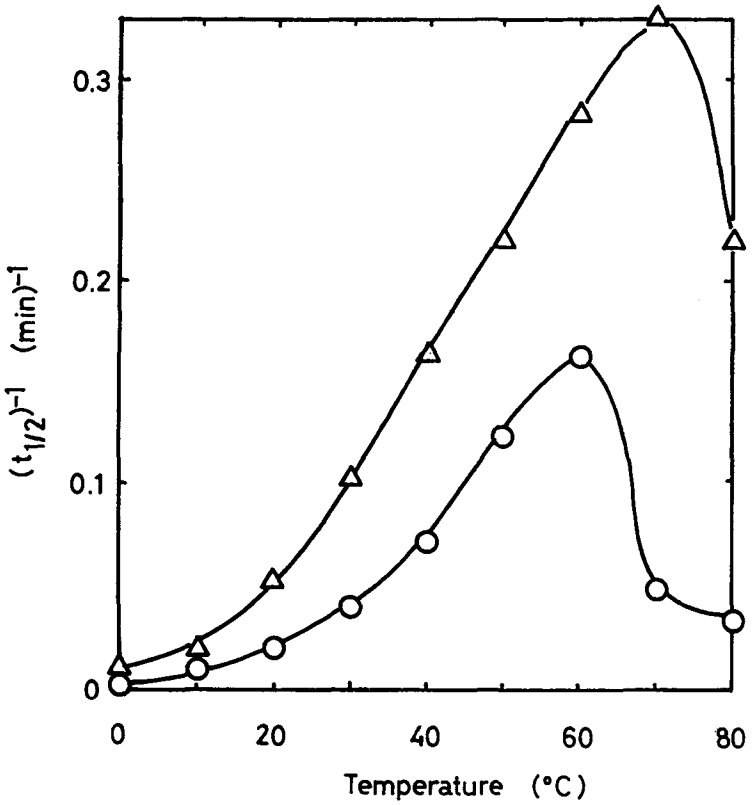

Fig. 5. Effect of temperature on the hydrolysis of PP. Porcine meat homogenate was prepared by the same procedure as described in the legend of Fig. 4. After measurement of pH, the homogenates were incubated at 0 to 80°C. ○, pH 5.6; △, pH 7.0.

TABLE 2. Hydrolysis of Polyphosphates in experimental Sausages

Sausage	A			B			C		
pH of emulsion	5.9			6.2			6.0		
Incubation temp (°C)	15	50	70	15	50	70	15	50	70
Incubation time (min)	Remaining PP (μmol/g meat)								
0	12.8	11.6	12.9	12.4	12.4	12.0	11.2	11.2	11.2
15	—	9.4	—	—	10.4	—	—	4.1	—
30	—	8.3	11.2	—	9.6	11.6	—	2.6	7.2
60	12.7	7.8	11.3	11.9	8.7	10.9	7.6	1.1	6.0
90	—	—	11.1	—	—	11.0	—	—	—
120	12.9	—	—	11.9	—	—	3.6	—	—

tion if blank containing glucose and G-6-P DHG is kept for long time after preparation. However, if glucose or G-6-P DHG is added to the assay mixture just before incubation, this phenomenon does not appear. The reason for this abnormal increase in optical density of blank is not clear. However, this modified method appears to quantitatively estimate inorganic PP concentrations and is not influenced by the presence of meat homogenates. Therefore, we used this enzymic procedure to determine the PP concentration in meat homogenates or sausage emulsions.

Figs. 2 and 3 show that PP added to meat homogenates was hydrolyzed during incubation. The rate of the hydrolysis depends on incubation temperature and pH value of the homogenate. NERAAL and HAMM[15~17] reported about tripolyphosphatase (TPase) and PPase activities in bovine longissimus muscle: The TPase activity increased, whereas the PPase activity decreased during the first two days post-mortem due to the drop in pH. The optimum pH for the activity was 5.7 for TPase and 7.0 for PPase[18]. We also found that the PPase activity in fresh meat was higher than the activity in long-time stored meat. However, in regard to the effect of pH, our results are at variance with those of NERAAL and HAMM[18]. As shown in Fig. 4, two pH optima in acid and neutral regions were found for PPase activity in both rabbit and porcine meat. So far many PPases have been reported from various sources, which have their pH optima in acid, neutral, and/or alkaline regions for mxaimal activity. Among them several homogenates of tissue such as potato[19] and bone[20] showed two or three pH optima for PPase activities. Therefore, it does not seem abnormal that meat homogenate has two pH optima. The presence of two pH optima indicates the presence of at least two PPases in muscle homogenate.

From the results described above, it was expected that the polyphosphates added to sausage emulsion would be hydrolyzed during processing. We made three experimental sausage emulsions, which contained about 2% NaCl and 0.3 to 0.5% commercial blend of polyphosphates or Na-PP (Table 1). If the emulsion contained only Na-PP, the latter is hydrolyzed rapidly. On the other hand, when a polyphosphate blend containing about 50% Na-PP and 50% other polyphosphates is used, the PP concentration decreased slowly. This is because the polyphosphates with longer chains are hydrolyzed to PP by some other polyphosphatases such as TPase[15]. At 50°C, the rate of hydrolysis of PP was the highest. This is reasonable from the results shown in Fig. 5 and pH of the emulsions (Table 2). PPase activity was higher at 50°C than at 70°C around pH 6. If the pH of emulsions were about 7, PP would be hydrolyzed more rapidly at 70°C. Usually pH of

sausage emulsions is between 5 and 6. In practice, emulsions are stored in a cold room briefly before making sausages, they are then smoked at about 50°C for 30 to 60 min, and cooked at 70 to 80°C for 30 to 90 min depending on the size. Our results show that if only Na-PP was used in sausage emulsions as a food additive, it would be hydrolyzed almost completely during the usual processing operations. However, the amount of the polyphosphates that remains in the emulsion system is not important, because it is only a certain amount of PP that is required to dissociate the actomyosin complex during making of emulsions[21], thereafter even the presence of PP is not vital in the sausages. It would be better for health of human beings that the products contain as little polyphosphates as possible since sequestrating ability of polyphosphates for calcium are high at neutral pH[22].

Summary

By using a modified enzymic method, inorganic pyrophosphate can be determined quantitatively and rapidly in meat homogenates. Inorganic pyrophosphate added to meat homogenates was hydrolyzed rapidly by endogeneous pyrophosphatases of meat. The hydrolytic activity depend on pH and the enzyme content in the homogeneates as well as on the presence of usual enzymes. The pyrophosphatase activity showed two pH optima at pH 5.6 and 7.

Even in experimental sausage emulsions, pyrophosphate was hydrloyzed almost completely after keeping under the similar conditions as used in usual processing. On the other hand, if a polyphosphate blend containing sodium pyrophosphate was used in the experimental sausage emulsions, the concentration of pyrophosphate was not decreased so rapidly when sodium pyrophosphate was used alone.

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