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Aromatic amino acid biosynthesis and production of related compounds from *p*-hydroxyphenylpyruvic acid by rumen bacteria, protozoa and their mixture

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Summary. Aromatic amino acid biosynthesis and production of related compounds from *p*-hydroxyphenylpyruvic acid (HPY) by mixed rumen bacteria (B), protozoa (P), and their mixture (BP) in an in vitro system were quantitatively investigated. Microbial suspensions prepared from mature, fistulated goats fed Lucerne (*Medicago sativa*) cubes and a concentrate mixture were anaerobically incubated at 39°C for 12 h. Tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp) and other related compounds in both supernatants and hydrolyzates of all incubations were analyzed by HPLC. Large amounts of Tyr (27.0, 47.0 and 50.8% of disappeared HPY in B, P and BP, respectively) were produced from 1 mM HPY during a 12-h incubation period. The formation of Tyr in P was 1.8 and 1.6 times higher than those in B and BP, respectively. Appreciable amounts of Phe (3–12% of the disappeared HPY) and Trp (2–10% of the disappeared HPY) were also produced from HPY in B, P, and BP. Phe synthesis in B and P was almost similar but Trp synthesis in B was 1.8 times higher than that in P. The biosynthesis of both Phe and Trp from HPY in BP was higher than those in B plus P. A large amount of *p*-hydroxyphenylacetic acid (about 45% of the disappeared HPY) was produced from HPY in B which was 1.9 times higher than that in P. *p*-Hydroxybenzoic acid produced from HPY in P was 1.6 times higher than that in B. Considerable amounts of phenylpropionic acid, phenyllactic acid, and phenylpyruvic acid (2–6% of the disappeared HPY) were produced only in B.

Keywords: Aromatic amino acid – *p*-Hydroxyphenylpyruvic acid – Rumen bacteria – Rumen protozoa

Introduction

Aromatic amino acids are essential to higher animals and are generally synthesized from glucose by a shikimate pathway in bacteria as well as in plants

(Bohm, 1979). However, shikimic acid is a poor precursor of the aromatic amino acid in rumen bacteria, presumably owing to its slow entry into the bacteria (Kristensen, 1974). It was found that different aerobic microorganisms, including *Aerobacter aerogenes* (Cotton and Gibson, 1968), *Pseudomonas aeruginosa* (Patel et al., 1977), *Bacillus subtilis* (Nester and Jensen, 1966), *Saccharomyces cerevisiae* (Lingens et al., 1966), *Neurospora crassa* (Baker, 1968), and different species of Coryneform bacteria (Fazel and Jensen, 1979) converted prephenic acid to *p*-hydroxyphenylpyruvic acid (HPY) and then transaminated to produce tyrosine (Tyr). It was reported that phenylalanine (Phe) and tryptophan (Trp) could be readily produced from phenylpyruvic acid (PPY) (Amin and Onodera, 1997) and indolepyruvic acid (IPA) (Okuuchi et al., 1993; Mohammed et al., 1999), respectively, by rumen bacteria (B), protozoa (P), and their mixture (BP). In the case of Tyr anabolism, Scott et al. (1964) observed that 27% of [U-^{14}C]*p*-hydroxyphenylacetic acid (HPA) was converted to Tyr after 1 h in vitro incubation with mixed rumen microorganisms. Kristensen (1974) suggested that in mixed rumen bacteria, HPA was reductively carboxylated to produce HPY and then transaminated to produce Tyr, though during his study the formation of HPY was not investigated and no information is available on Tyr production in rumen bacteria directly from HPY. On the other hand, biosynthetic pathway for Tyr from HPA or HPY in rumen protozoa is completely unknown, and it has been reported that both rumen bacteria and protozoa synthesized Tyr from Phe (Khan et al., 1999). The present study was conducted to quantitatively investigate the in vitro synthesis of Tyr and production of related compounds from HPY by B, P, and BP using a convenient HPLC method (Khan et al., 1998).

Materials and methods

Collection of rumen contents and preparation of microbial suspensions

Rumen contents were collected before morning feeding of three fistulated goats (Japanese native breed, live weight 40 ± 5 kg), fed on a daily ration consisting of Lucerne (*Medicago sativa*) hay cubes (23 g DM/kg $BW^{0.75}$) and concentrate mixture (8 g DM/kg $BW^{0.75}$) provided in two equal portions at 9 am and 5 pm. The goats were housed in individual pens with a good ventilation system and they had ad libitum access to fresh water.

Rumen contents were strained through four layers of surgical gauze into a separating funnel which was gassed with a mixture of 95% N_2 and 5% CO_2. The strained contents were then incubated at 39°C for about 60 min to allow feed debris to float. The lower liquid portion was separated, mixed well and a portion was used as BP suspension. The suspensions of B and P were prepared from the remaining lower liquid portion according to Onodera et al. (1992).

Incubation and sample treatments

Microbial suspensions (20 ml) were anaerobically incubated with and without 1 mM HPY (Wako Pure Chemical Industries, Osaka, Japan) as a substrate in a water bath at 39°C for 12 h. All incubations contained 0.5 mg/ml of rice starch for energy source, and only P

suspensions always included 0.1 mg/ml each of chloramphenicol, streptomycin sulfate and penicillin G potassium to suppress the growth of contaminating bacteria. Samples were collected (0.5 ml) at 0, 6 and 12 h, mixed with an equal volume of 5% (v/v) perchloric acid (Neckers et al., 1981) for deproteinization, and centrifuged at 27,000 g for 20 min at 4°C. The supernatant fluids were filtered through a membrane filter (pore size, 0.45 μm). Pellets were hydrolyzed with 4 M methansulphonic acid at 160°C for 45 min (Chiou and Wang, 1988). Samples were diluted 20 times and filtered through a membrane filter (pore size, 0.45 μm) for HPLC analysis.

Analytical method

Samples were analyzed by HPLC (Khan et al., 1998). The values of all the components found in the supernatants and hydrolyzates were expressed as the means of nine determinations and standard deviations of the differences between incubation with and without substrates. Protozoal numbers of P and BP suspensions were counted according to Onodera et al. (1977) and observed that the protozoal compositions were 158.2 and 96.5, 11.6 and 5.3, and 2.8 and 1.6 $\times$ 10^4/ml cells for *Entodinium*, *Diplodinium*, and *Dasytricha*, respectively. Microbial nitrogen (MN) of B, P, and BP suspensions were determined by Kjeldahl method (AOAC, 1990). Average microbial nitrogen (mg MN/ml) in B, P, and BP suspensions were 1.212 $\pm$ 0.079, 1.189 $\pm$ 0.131, and 2.070 $\pm$ 0.104, respectively.

Results and discussion

Disappearance of HPY

The disappearance of HPY (initial concentration, 1 mM) in B, P, and BP suspensions during 6 and 12 h incubation periods were 84.5 and 100.6%, 70.4 and 99.8%, and 91.9 and 100.8%, respectively. Thus, almost all of the substrate in each suspension was consumed by the microorganisms within 12 h. After 6 h incubation disappearance rate of HPY in B (116 μmol/g MN/h) was 1.2 times higher than that in P. The disappearance rates of HPY from the incubations of B, P, and BP observed in this study are the first evidence.

Synthesis of Tyr from HPY

A large amount of Tyr was synthesized from HPY by B, P, and BP. During 6 and 12 h incubation in 1 mM HPY, Tyr increased in the supernatant of B, P, and BP suspensions (Fig. 1). In B and BP, most of the Tyr produced from HPY was found after 6 h, but in P, it increased with the incubation time up to 12 h. As shown in Fig. 2, a considerable amount of Tyr increased in the hydrolyzates of P at 6 h and reduced at 12 h of incubation, indicating that a part of Tyr found in the supernatant of P was liberated from their cell proteins (Onodera and Kandatsu, 1970; Morgavi et al., 1993). A large amount of Tyr produced from HPY was utilized efficiently for bacterial cell protein synthesis (Fig. 2) (Broderick et al., 1991; Armstead and Ling, 1993; Ling and Armstead, 1995).

The net production of Tyr in B, P, and BP was calculated from the increment of Tyr in the supernatant (Fig. 1) and simultaneous incorporation

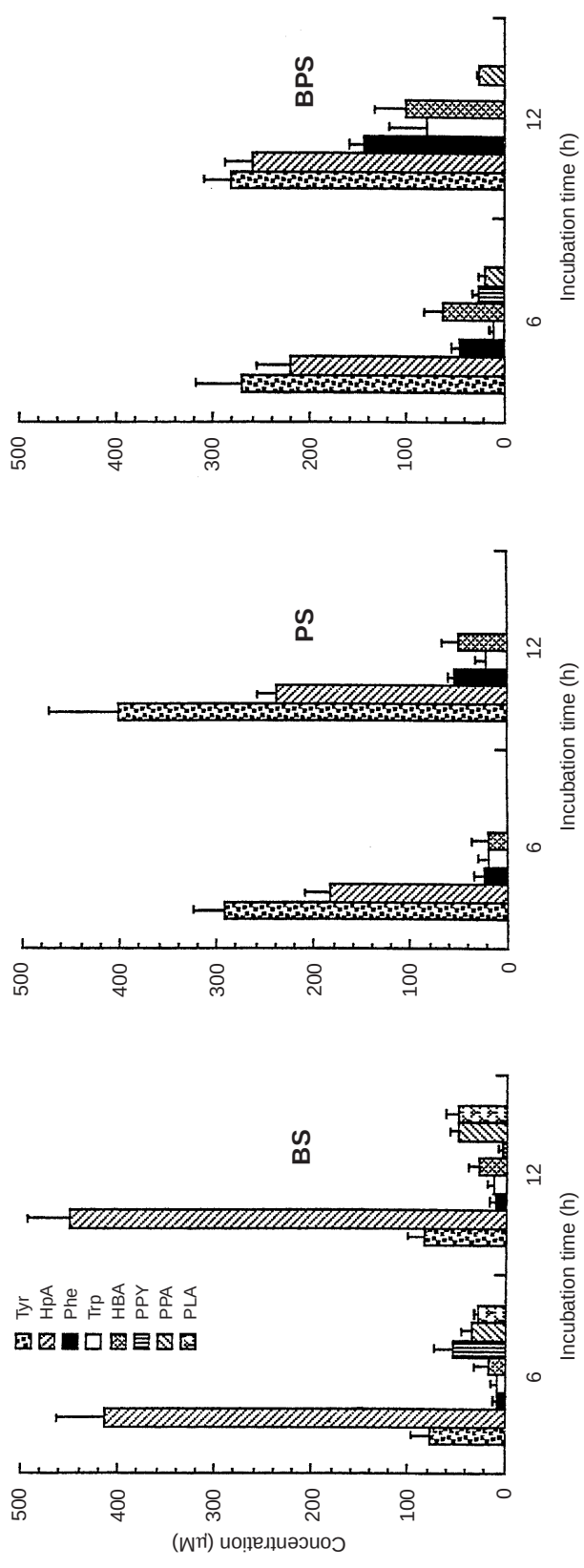

Fig. 1. Formation of tyrosine (*Tyr*), phenylalanine (*Phe*), tryptophan (*Trp*), *p*-hydroxyphenylacetic acid (*HPA*), *p*-hydroxybenzoic acid (*HBA*), phenylpropionic acid (*PPA*), phenyllactic acid (*PLA*) and phenylpyruvic acid (*PPY*) in the supernatants of rumen bacteria (*BS*), protozoa (*PS*) and their mixture (*BPS*) during a 12-h incubation. Average microbial nitrogen (mgMN/ml) in B, P, and BP suspensions were 1.212 ± 0.079, 1.189 ± 0.131, and 2.070 ± 0.104, respectively. Protozoal compositions ($\times 10^4$ cells/ml) in P and BP were 158.2 and 96.5; 11.6 and 5.3, and 2.8 and 1.6 for *Entodinium*, *Diplodinium*, and *Dasytricha*, respectively

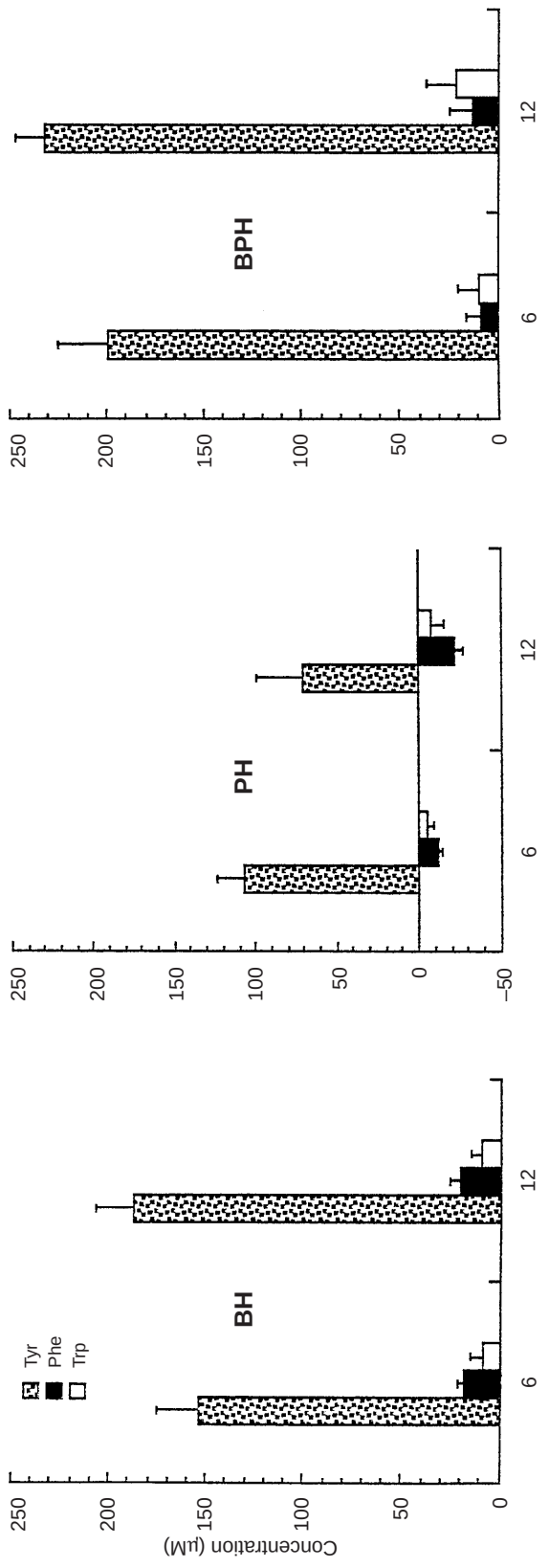

Fig. 2. Incorporation of tyrosine (*Tyr*), phenylalanine (*Phe*) and tryptophan (*Trp*) in hydrolyzates of mixed rumen bacteria (*BH*), protozoa (*PH*) and their mixture (*BPH*) during a 12-h incubation. Conditions of microbial suspensions are the same as shown in Fig. 1

into microbial cells (Fig. 2). It was observed that about 23.2 and 27.2%, 39.7 and 46.9%, and 46.8 and 51.2% of the HPY was converted to Tyr during 6 and 12 h incubation in B, P and BP suspensions, respectively (Fig. 3). Tyr synthesis from HPY in P during a 12 h-incubation period (395 μmol/g MN) was 1.8 and 1.6 times higher than those in B and BP, respectively. The quantitative study of the production of Tyr from HPY by B, P, and BP has been reported for the first time from this study. The production of Trp (Okuuchi et al., 1993; Mohammed et al., 1999) and Phe (Amin and Onodera, 1997) from IPA and PPY, respectively, by B, P, and BP have been demonstrated and in Trp biosynthesis rumen protozoa have been revealed to have high aminotransferase activity (Okuuchi et al., 1993).

Synthesis of Phe from HPY

The concentrations of Phe increased in the supernatant of B, P, and BP (10, 54 and 143 μM, respectively) (Fig. 1). Portions of Phe produced from HPY were also incorporated into the cells of B and BP. On the other hand, P released more Phe in the supernatant than incorporated (Fig. 2). The net production of Phe from HPY in B, P, and BP was calculated and presented in Fig. 3. Phe formation from HPY in a 12-h incubation in B (24 μmol/g MN) and P (26 μmol/g MN) was almost similar, but it was 2.9 fold higher in BP (69 μmol/g MN). About 3% of the disappeared HPY was converted to Phe in both B and P, but in case of BP about 12% was converted. The formation of Phe from HPY by B, P, and BP found in this study is the first demonstration of its kind. Kristensen (1974) observed a considerable amount of radioactive Phe after 1 h incubation of [U-^{14}C]HPA with mixed rumen bacteria. During the present study, the highest production of Phe from HPY in BP may be due to a complicated interaction between B and P, resulting in a higher value than the mechanical sum of the single values of B and P.

Synthesis of Trp from HPY

After 6 and 12 h incubation with HPY, Trp increased in both supernatants and hydrolyzates of B and BP, but in case of P, only in the supernatant (Figs. 1 and 2). It was indicated that rumen bacteria efficiently utilized Trp produced from HPY for their cell protein synthesis and protozoa liberated much Trp in the media. On the other hand, 2.2, 1.1 and 10.1% of the HPY were converted to Trp in B, P, and BP, respectively, at a 12-h incubation period (Fig. 3). The formation of Trp from HPY in B (18 μmol/g MN) was 1.8 times higher than that in P. The production of Trp in BP (49 μmol/g MN) was higher than that in B plus P. It can be explained that BP rapidly degraded HPA produced from HPY and then converted to Trp. During the Trp biosynthesis from IPA, Mohammed et al. (1999) observed that the formation rates of Trp in B and P were similar at 12 h incubation, although Okuuchi et al. (1993) found a higher aminotransferase activity in P than that in B. Kristensen (1974) suggested that after 1 h incubation a very small amount of radioactive Trp was produced

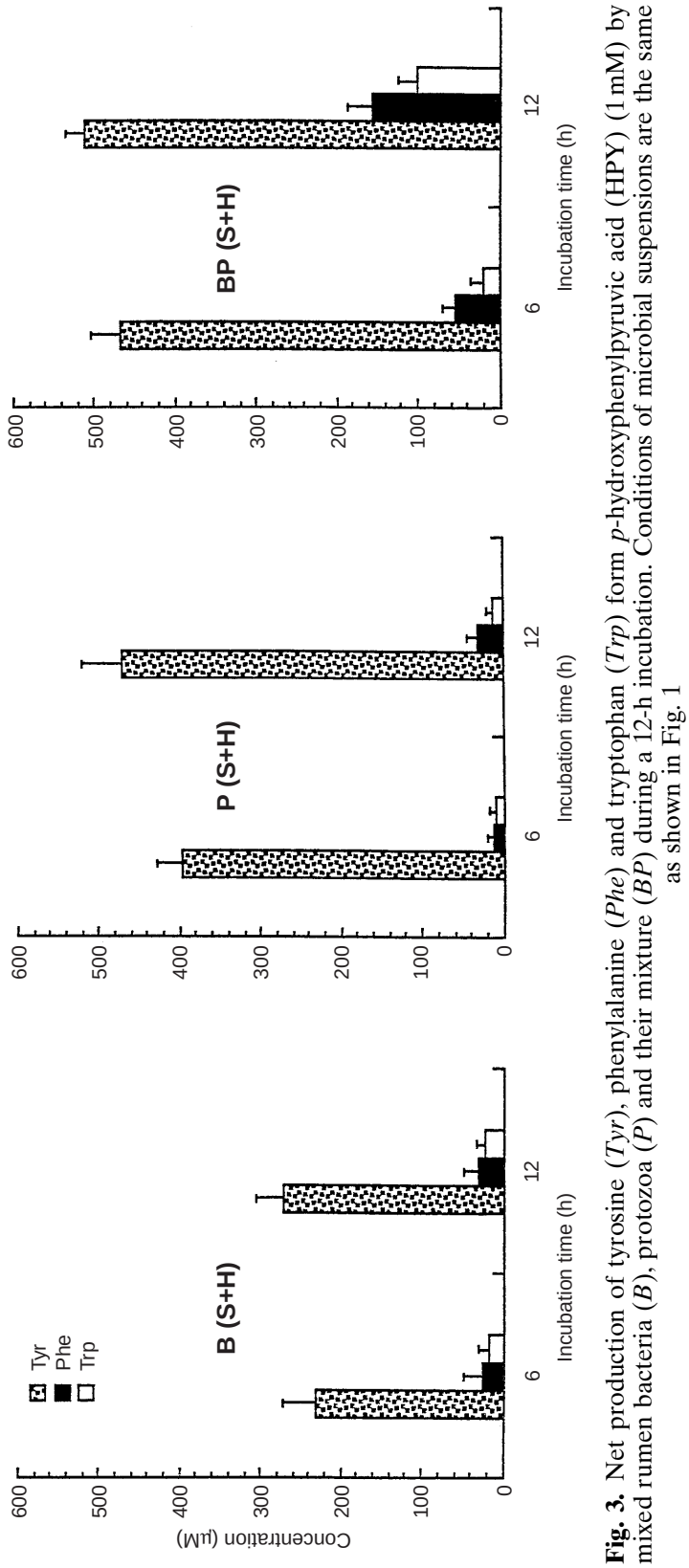

Fig. 3. Net production of tyrosine (*Tyr*), phenylalanine (*Phe*) and tryptophan (*Trp*) form *p*-hydroxyphenylpyruvic acid (HPY) (1 mM) by mixed rumen bacteria (*B*), protozoa (*P*) and their mixture (*BP*) during a 12-h incubation. Conditions of microbial suspensions are the same as shown in Fig. 1

from [U-^{14}C]HPA by mixed rumen bacteria. The formation of Trp from HPY by B, P, and BP has been evidenced for the first time in this study.

Production of HPA from HPY

A large amount of HPA was produced from HPY in B, P, and BP after 6 and 12 h incubation (Fig. 1). It was observed that 41.2 and 45.0, 18.3 and 23.8, and 22.1 and 25.4% of the HPY were converted to HPA in 6 and 12 h incubation with B, P, and BP, respectively. The production of HPA in B (340.3 and 371.0 μmol/g MN in 6 and 12 h incubation, respectively) was 2.0 and 3.1 times higher than those in P and BP, respectively. The lower value of HPA production in BP can be assumed that HPA produced by a rapid degradation of HPY in BP and again degraded to form aromatic amino acids or other related compounds like HBA (Figs. 1 and 3). Formation of radioactive HPA from L-[U-^{14}C]Tyr was also observed in mixed rumen microorganisms (Scott et al., 1964). Phenylacetic acid and indoleacetic acid were produced from PPY and IPA, respectively by B, P and BP (Okuuchi et al., 1993; Amin and Onodera, 1997; Mohammed et al., 1999). The production of HPA from HPY is confirmed for the first time in this experiment.

Production of HBA from HPY

After 6 and 12 h incubation, 1.8 and 2.8, 1.9 and 5.0, and 6.2 and 10.1% of HPY were converted to HBA by B, P, and BP, respectively. The production of HBA in P (16 and 42 μmol/g MN in 6 and 12 h incubation, respectively) was 1.6 times higher than that in B. The formation of HBA from HPY in BP (30 and 49 μmol/g MN in 6 and 12 h incubation, respectively) was 2.2 and 1.4 times higher than those in B and P, respectively. The higher value of HBA in BP might have been caused by the re-degradation of other products produced from HPY. Benzoic acid formations from Tyr by mixed rumen microorganisms (Scott et al., 1964), and from PPY by B, P, and BP (Amin and Onodera, 1997) were previously demonstrated, but the productions of HBA from HPY by B, P, and BP, as indicated in this experiment, are being reported for the first time.

Production of PPA, PLA and PPY from HPY

A considerable amount of PPA was produced from HPY in B (3.3 and 4.9% of HPY in 6 and 12 h incubation, respectively) and BP (1.9 and 2.5% of HPY in 6 and 12 h incubation, respectively), being the first evidence, although the formations of PPA from Tyr by mixed rumen microorganisms (Scott et al., 1964) were previously reported.

It was found that 2.6 and 4.8% of the HPY were converted to PLA only by B after 6 and 12 h incubation, respectively. The production of PLA from PPY in B was previously observed (Amin and Onodera, 1997), but the ability of B

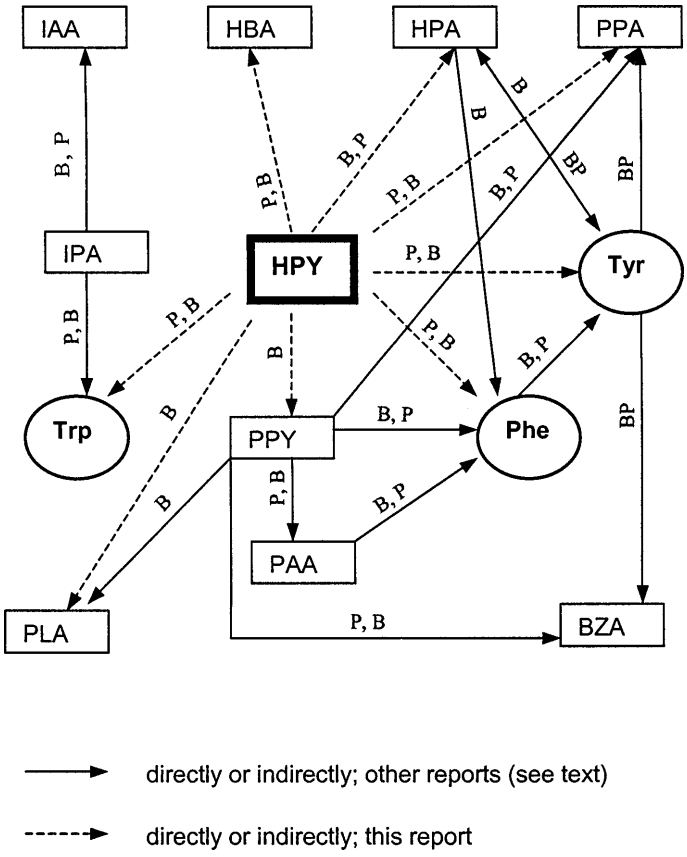

Fig. 4. Possible metabolic pathways around *p*-hydroxyphenylpyruvate (*HPY*) in rumen bacteria (*B*), protozoa (*P*) and their mixture (*BP*). HPA, *p*-hydroxyphenylacetic acid; *PPY*, phenylpyruvic acid; *Tyr*, tyrosine; *Phe*, phenylalanine; *PAA*, phenylacetic acid; *PPA*, phenylpropionic acid; *BZA*, benzoic acid; *Trp*, triptophan; *IPA*, indolepyruvic acid; *IAA*, indoleacetic acid; *PLA*, phenyllactic acid; *HBA*, *p*-hydroxybenzoic acid

to produce PLA from HPY was first demonstrated for the first time in the present study.

As shown in Fig. 1, considerable amounts of PPY were produced from HPY at a 6-h incubation with B and BP (5.2 and 2.4% of HPY, respectively), but the amount of PPY decreased after 12-h incubation period. This is the first evidence of PPY production from HPY by B and BP, although PPY is unstable specially in acidic medium of the analytical method (Khan et al., 1998).

After considering the previous reports published on various aspects of aromatic amino acid biosynthesis and production of related compounds as well as the new findings of the present investigations a possible new schematic pathway around of HPY in rumen bacteria, protozoa and their mixture has been proposed and shown in Fig. 4.

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