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The Isolation of Phosphorylhomoserine from Trichloroacetic Acid Extracts of *Lactobacillus casei*

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Free phosphorylhomoserine has been isolated in considerable amounts from trichloroacetic acid extracts of *Lactobacillus casei*. The mechanism for the formation of the substance is discussed. Some phosphopeptides present in the extract have also been purified and their amino acid composition determined.

In a recent communication from this laboratory evidence was presented for the occurrence in the trichloroacetic acid soluble fraction of *L. casei* of a free phosphorylated amino acid of unknown composition [1]. This paper describes the identification of this compound as phosphorylhomoserine (HserP) *.

EXPERIMENTAL

Preparation. Lactobacillus casei (7461) was cultivated in a medium with the following composition [2]: 10 g of Dextropur **, 10 g of $NaOCOCH_3 \cdot 3H_2O$, 10 g of Bactopeptone *, 1 g of yeast extract **, 25.4 ml of liver extract ***, 0.5 ml of salt mixture **** per 1000 ml of medium at pH 6.8. For large scale cultivation of radioactive labelled bacteria (120 l) the necessary ingredients were first dissolved in 5 l water and heated at 100°C for about 15 min. After cooling a dark precipitate was removed by filtration. The filtrate was evenly distributed in forty 6 liter Erlenmeyer flasks and subsequently water was added to a final volume of 3 l per flask. After adjusting the pH to 6.8, sterilization was accomplished by autoclaving the flasks 15 min. at 118°C.

*Abbreviations: HserP = O-phosphorylhomoserine; Hser = homoserine; ^{32}P = inorganic radioactive phosphate; TCA = trichloroacetic acid; UV = ultra violet; AMP = adenylic acid; MurP = O-phosphorylmuramic acid; ThrP = O-phosphorylthreonine; ATP = adenosine triphosphate; ADP = adenosine diphosphate; P_i = inorganic phosphate; Thr = threonine.

** Glucose manufactured by Corn Products Co., New York.

* Parke, Davis and Co.

** Difco Laboratories.

*** 100 g of Difco Liver was dissolved in 1330 ml and kept at 50° for one hour and subsequently at 80° for 5 min. After filtration and concentration the filtrate was lyophilized. This material was dissolved to contain 15 mg per ml.

**** 4 g of $MgSO_4.H_2O$, 1 g $FeSO_4.7\ H_2O$, 1 g of NaCl, 0.1 g of $MnSO_4.H_2O$ were dissolved to 100 ml.

Inocula for large scale cultivation was prepared by transferring a small amount of growth from a stab culture to two 10 ml flasks, each containing 5 ml of medium. The amount of inocula was increased in the five subsequent steps as previously described [3]. 300 ml suspension from step 5 was then added to each 6 l flask containing 3 l sterilized medium. 1 mC radioactive phosphate per liter medium was added at the same time. Previously it had been shown that the presence of as much as 100 mC per liter medium had no demonstrable effect on growth or acid production over a period of 72 h [4]. Incubation was carried out in an air incubator for 17 h at 37°C. In order to increase the utilization of ^{32}P added to the medium inorganic phosphate was excluded from the salt mixture since preliminary experiments had shown that the phosphorus present in Bactopeptone and liver furnished sufficient total phosphorus (0.005 %) for normal growth.

At the end of the cultivation the radioactive bacteria were separated from the medium in Sharples centrifuges enclosed in a small stainless steel house with a perspex window and with separate effective ventilation. The usual yield of fresh bacteria from a single 120 liter cultivation was about 500–600 g. This material was washed two times with 10 volumes of cold 10 % TCA, each time for about 15 min. The supernatant fluids from the extracted and centrifuged cell suspensions were shaken with ether several times to remove the TCA. The solution was evaporated to a volume of about 300 ml, again shaken several times with ether, and the pH adjusted to 6–7 by addition of a few drops of 1 N NaOH. The concentrate was then run through a 90 × 3 cm Dowex 1 (2 % DVB) formate column mainly according to Hurlbert *et al.*[5] A radioactive peak with inconsiderable UV absorption at 260 or 280 mμ was observed (Fig. 1 in Ref.[6]) located in the position between AMP and inorganic phosphate and eluted between 0.5 M and 2.5 M formic acid concentration. The TCA soluble fraction from each batch of bacteria containing the radioactive, non-UV-absorbing peak was concentrated *in vacuo* to a small volume with repeated additions of water to remove excess formic acid. The concentrated solution was run through a Dowex 50 (8 % DVB) column with 0.01 N HCl (column dimensions 2 cm × 40 cm for a 120 liter culture). Two peaks containing ninhydrin-positive material were eluted following the inorganic phosphate, the first after about 1.0 column volume and the second after about 2.3 column volumes of eluate (effluent volume/resin volume). Fig. 1 in Ref.[1] pictures a typical result.

The material from the second peak which contained the new phosphorylated amino acid was collected and excess of acid removed as described above. Each sample of material was again run through a small Dowex 1 (2 % DVB) formate column (0.9 cm × 50 cm) and eluted mainly according to Hurlbert *et al.*[5] with a gradient 0 → 1 M formic acid and with a volume of 300 ml of the mixer. A large peak appeared when the acidity in the eluate reached a concentration corresponding to 0.60 M formic acid or in the same position as MurP [3]. In Fig. 3 in Ref.[1] the elution curve from a typical experiment is given. The total dry weight of bacteria from five large scale preparations was 650 g. The total weight of the new TCA-soluble compound from the five experiments amounted to 55 mg. Several attempts to crystallize the substance from water, water-methanol or water acetone mixtures failed. The phosphorylated compound was rather soluble in these solvents.

In order to repeat these experiments with larger amounts of material a large scale cultivation of the microorganisms were carried out in a 1000 liter tank on the medium described before *. The yield of fresh bacteria was about 8 kg. The material was washed with cold TCA and the TCA extracts shaken with ether and concentrated *in vacuo* as described before. The concentrated TCA extracts from the unlabelled batch of microorganisms were mixed with a TCA extract from a 120 liter cultivation of labelled bacteria. The mixed extracts were then filtered into five Dowex 1 formate columns (50 × 6.5 cm) and eluted mainly according to Hurlbert *et al.*[5] The eluate was collected in fractions of about 70 to 80 ml every 15 min.

The elution was started with water and about 48 fractions were collected. In the system which followed, a 1700 ml mixing volume was used. The four elution ranges which approximated 1 N formic acid, 4 N formic acid, 0.2 M ammonium formate in 4 N formic acid and 0.4 M ammonium formate in 4 N formic acid were obtained by changing the reservoir to contain these solutions at tubes 48, 200, 600 and 850. Altogether 1000 fractions were collected in each series.

* The cultivation was carried out in the pilot plant of the Department of Bacteriology, Karolinska institutet, Stockholm.

Analytical methods. The nucleotides were assayed as previously described [6]. The radioactivity of the different fractions from the column chromatography of the TCA soluble nucleotides was measured in glass cups with the L.K.B. Robot Scaler. Scanning of the paper chromatogram of HserP, phosphopeptides and nucleotides was made according to Ågren *et al.*[7]

The amino acid composition of phosphopeptides was determined by two-dimensional chromatography [8]. This method was used together with one-dimensional chromatography in 0.1 N ammonium acetate-ethanol (3:7 by vol.), isobutyric acid-ammonia-water (79:1:25 by vol.), butanol-acetic acid-water (4:1:5 by vol., solvent phase) [9]. Phosphopeptides were hydrolyzed with 2 N HCl for 20 h at 120°C in sealed tubes. For chromatography Whatman No. 1 papers were used, and for ionophoresis Whatman No. 3, where the solvents were either a 0.1 M solution of pyridine-acetic acid at pH 5 or 1 N acetic acid.

Synthesis of DL-*phosphorylhomoserine.* The barium salt of DL-phosphorylhomoserine was synthesized according to Levene and Schormüller [10] by phosphorylation of homoserine with phosphoric acid and phosphoric anhydride. Barium was removed by electrodialysis and the substance purified by running through a small Dowex 50 column (55 cm × 0.9 cm) by elution with 0.01 N HCl. The effluent fluid in the tubes was tested for ninhydrin-reacting substance. DL-Phosphorylhomoserine crystallized out of water solutions. The yield was about 5 % of the theoretical. (Found: C 24.9; H 4.88; N 7.16; P 15.0. Calc. for $C_4H_{10}O_6NP$ (199.1): C 24.1; H 5.06; N 7.04; P 15.6).

Dephosphorylation of HserP was performed in sealed glass tubes with 2 N HCl at 120°C for 20 h. Still some phosphorylated product was present and appeared on chromatograms. Hser in the hydrolyzed samples was then purified by preparative paper electrophoresis in 1 N acetic acid. Hser was washed out from the paper strip with distilled water.

RESULTS

Separation of phosphorylhomoserine from phosphopeptides

Also in the 1000 liter cultivation the radioactive peak with inconsiderable UV absorption, containing the desired material was located in the same place of the elution curve as in small scale preparations, *viz.* in the position between AMP and the next large UV-peak (Fig. 1, Ref.[6]). Other parts of the elution diagrams were quite similar to that pictured in this figure.

The ether extracted TCA soluble fraction from the five column eluates containing the peak without UV absorption was concentrated *in vacuo* to a

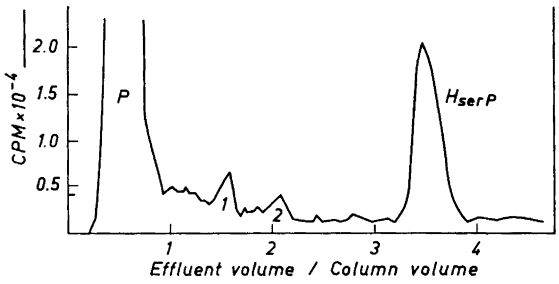

Fig. 1. TCA soluble material collected between 0.7 and 2.5 M formic acid solution on a Dowex 1 column and afterwards separated on a Dowex 50 column. The radioactivity curve is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube.

small volume with repeated additions of water to remove excess of formic acid. The concentrated solution was run through a Dowex 50 W (X-8, 200—400 mesh) column with 0.01 N HCl (column dimensions 3 cm × 50 cm). 10 ml fractions were collected at a rate of 0.66 per minute. In Fig. 1 the elution curve is given. A comparison with a similar elution diagram from small scale cultivations (Fig. 1, Ref.[1]) showed some significant differences. In the present diagram two small peaks containing ninhydrin-positive material of phosphopeptide nature were eluted while in earlier experiments only one such peak was observed. Further, these peptide peaks as well as HserP were delayed on the elution curve. Systematic investigations have shown this to be a general phenomenon when Dowex 50 W (white) is used.

Analysis of the phosphopeptide fractions. In the earlier paper it was demonstrated that the single phosphopeptide peak was not homogeneous. A ionophoretic control (pyridine-acetate buffer at pH 5) showed two radioactive peaks. The small amounts of material did not allow rechromatography of this fraction on a second Dowex column. After hydrolysis, two-dimensional paper chromatography of the material showed at least six spots corresponding to the position of aspartic acid, glutamic acid, serine, threonine, alanine and leucine or isoleucine, all members of the phosphopeptide isolated from the enzymatic active center of chymotrypsin or phosphoglucomutase [11].

Consequently a new attempt was made to investigate the homogeneity of the two phosphopeptides which now were separated already on the first Dowex 50 column. The material from peaks 1 and 2 was collected and excess HCl removed as described above. Each sample of material amounted only to a few mg and was run through small Dowex 1 (2 % DVB) formate columns (0.9 cm × 20 cm) and eluted mainly according to Hurlbert *et al.*[5] with a gradient 0→1 M formic acid and with a volume of 150 ml in the mixer. In Figs. 2 and 3 the elution curves are given. Obviously both of the two phosphopeptide fractions from the Dowex 50 column consisted of complex mixtures of peptides. The material from peak No. 1 was resolved into at least three major peaks, A, B and C, weighing from 0.2—1.5 mg which were collected for analysis. The last large peak eluted at 0.9 M formic acid concentration was inorganic phosphate. In ionophoretic controls of the homogeneity of the material from peak A in 1 N acetic acid a radioactive peak could not be found in the scanning

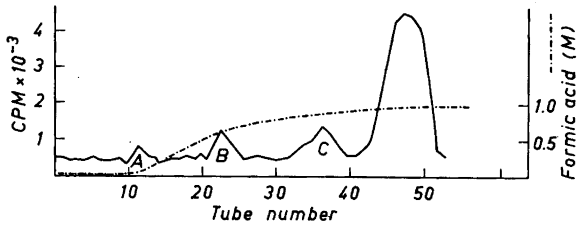

Fig. 2. Radioactivity curve of the material from peptide fraction No. 1, Fig. 1, separated on a Dowex 1 column. The radioactivity curve (the continuous line) is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

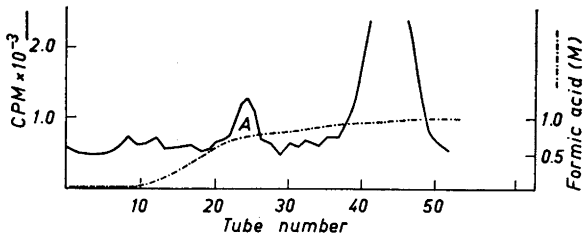

Fig. 3. Radioactivity curve of the material from peptide fraction No. 2, Fig. 1, separated on a Dowex 1 column. The radioactivity curve (the continuous line) is obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

of the electropherogram. After acid hydrolysis paper chromatographic analysis showed spots corresponding to the location of aspartic and glutamic acids, serine alanine and three other unidentified amino acids.

The electropherogram of fraction B, 1.5 mg, showed one radioactive and one smaller unlabelled ninhydrin-positive spot. The small amount of material did not allow purification by preparative paper electrophoresis. A two-dimensional chromatogram of the hydrolysate from B showed spots corresponding to the positions of aspartic and glutamic acids, serine, glycine, alanine, a spot with the same color and the same position as β-alanine and one unidentified spot below the glycine spot. The proportions of identified amino acids were other than in the hydrolysate of fraction A. A paper electropherogram of peak C (1.0 mg) showed that it consisted of two radioactive ninhydrin-positive spots too close to each other to allow a preparative electropherographic separation. The hydrolysate contained the same amino acids as in B but in other proportions.

The material from peak No. 2 from the Dowex 50 column gave an elution diagram from the Dowex 1 column as demonstrated in Fig. 3. Only the larger peak A eluted between 0.60 to 0.75 M formic acid was collected. The last large peak on the elution diagram was inorganic phosphate. The weight of A was 2.0 mg. A ionophoretic control of the homogeneity (1 M acetic acid) showed one radioactive and one unlabelled ninhydrin-positive spot well separated from each other. Accordingly, the rest of the material was purified by ionophoresis and elution of the radioactive section of the paper strip with distilled water. As control another section of similar size was also washed with water. Two-dimensional paper chromatography of the purified, hydrolyzed material showed spots corresponding to the position of aspartic and glutamic acids, serine, glycine, threonine, alanine, valine and isoleucine or leucine.

The isolation of phosphorylhomoserine. The material forming the large peak in Fig. 1 labelled HserP was collected and excess HCl removed as described above. The weight was 218 mg. The material was run through a small Dowex 1 (2 % DVB) formate column (0.9 cm × 20 cm) and eluted with a gradient 0 → 1 M formic acid and with a volume of 125 ml in the mixer. Only one large peak appeared between 0.60 and 0.80 M formic acid or in the same position previously found for the same material from small scale cultivations [1] and

also for MurP [3]. The weight of the material from the peak was 210 mg. Since the total dry weight of the bacteria was 1.100 g the yield was better than in the previous small scale preparations [1]. The slightly yellow coloured material was again purified chromatographically on a small Dowex 50 W (X-8, 200—400 mesh) column with 0.01 N HCl (column dimensions 0.9 cm × 55 cm). Only one large symmetrical peak appeared when 3.2 column volume of HCl had passed through or in the same position as in Fig. 1. The dry weight of material was 200 mg.

Identification and characterization

Elementary analysis of the isolated substance was already given in the previous paper. The figures indicated that the substance could be either phosphorylthreonine, previously isolated in this laboratory first from casein [12,13] and later from sheep liver protein [14] or the isomer phosphorylhomoserine. The position of the dephosphorylated amino acid on a two-dimensional paper chromatogram did not coincide with that of threonine. It could be suspected that the new compound was phosphorylhomoserine. With the larger amounts of material from the large scale cultivation the substance could be crystallized by slow evaporation from water solutions as needleshaped crystals.

Elementary analysis of C, H, and N were made by Mr W. Kirsten at this institute. Phosphorus was analyzed according to a modification of Martin and Doty [15].

$C_4H_{10}O_6NP$ (199.1)	Calc.	C 24.1;	H 5.06;	N 7.04;	P 15.6
	Found 1	25.6	4.40	7.12	15.0
	2	24.7	5.24	6.55	14.8
	3	24.7	—	7.07	15.8

Paper ionophoresis and paper chromatography. The ionophoretic migration of HserP in comparison to SerP and ThrP is shown in Table 1. The dephosphorylated compound showed the same mobility as serine and threonine at pH 5. Fig. 4 is a photograph of a chromatogram of the dephosphorylated compound and synthetic Hser running parallely. In the five solvents listed in a preceding paper [9] identical R_F values for the two compounds were obtained. The same result was obtained with 0.1 M ammonium acetate-ethanol (3:7). In several two-dimensional chromatograms the dephosphorylated compound and synthetic Hser only gave a single spot.

X-Ray studies. X-Ray powder photographs of the isolated compound and DL-HserP are given in Fig. 5. The two crystal preparations did not give identical diagram.

Table 1. The ionophoretic mobility of HserP in 1 N acetic acid compared with that of SerP and ThrP (the Pherograph of Wieland-Pfleidern). Time 105 min, 2800 V and 15 mA. + indicate that the substances move toward the anode. The distance measured to slowest part of the spots. Figures are given in cm.

Isol. HserP	Synt. HserP	SerP	ThrP
+ 18	+ 18	+ 21	+ 17

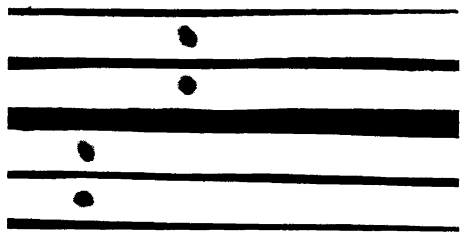

Fig. 4. Two one-dimensional chromatograms run with isobutyric acid-ammonia-water (below) and pyridine-isoamylalcohol-water (above) showing that the dephosphorylated compound gives a spot identical with synthetic homoserine.

Optical rotation. For HserP the following value was obtained $\alpha_D^{22.5}$ + 0.15° (water, *l*, 1; *c* 2.4) which corresponds to $[\alpha]_D^{22.5}$ + 6.25°; $[M]_D^{22.5}$ + 12.4°. M.P. 178° (decomp.).

DISCUSSION

From the results it seems to be proved that the isolated free phosphorylated amino acid in the TCA extracts of *L. casei* is HserP. So far it has not been possible to establish the presence in these extracts of free SerP recently found in the corresponding extracts of animal livers and kidneys [16]. A probable reason for the finding of free SerP in the organ extracts could be the recent observation of Ichihara and Greenberg [17]; using a crude enzyme preparation they presented evidence for the biosynthesis of serine from glyceric acid *via* SerP, which should be dephosphorylated to serine. If that was the single reason for the presence of free SerP in the organ extracts it could have been expected to find free SerP also in the microbial extracts. However, it is quite possible that the bacterial biosynthesis of serine might proceed according to other lines.

About in the same position as SerP on the elution diagram of the bacterical TCA extracts through the Dowex 50 column only two phosphopeptide frac-

Fig. 5. X-Ray powder diagram of HserP from *L. casei* (upper) and synthetic HserP (below). Guinier camera. Nickel-filtered copper *K*-radiation.

tions of complex composition could be observed. From one of these it was possible to purify a phosphopeptide which seemed to be homogeneous on paper electropherogram. It is of considerable interest that the amino acid composition of this peptide is the same as found by Koshland [11] and others [18-20] in the phosphopeptides from the "active site" of phosphoglucomutase and chymotrypsin. The reason for the presence of free peptides of this type in the TCA extracts of *L. casei* is at present not clear.

The optical rotation shows about the same values as SerP as well as ThrP, the latter with different sign [13,21]. Phosphorylserine: $[\alpha]_D^{23}$ + 7.2°; $[M]_{23}^D$ + 13.5. Phosphorylthreonine: $[\alpha]_{24}^D$ −7.4°; $[M]_{24}^D$ −14.6. The optically active and the racemic form of HserP did not give identical X-ray diagrams as was also the case with ThrP [13]. In the latter instance it was probable that the DL-forms were a conglomerate as has been found for the unphosphorylated amino acid [22].

Although this communication seems to be the first report of the isolation of free HserP in extracts of *L. casei* there has been some earlier evidence for a possible presence of the compound in similar extracts. Using ^{14}C labelled glucose it has been shown that homoserine while not a structural component of the bacterial protein is a precursor of threonine in *E. coli* [23]. Wotanabe *et al.*[24-26] using crude enzyme preparations showed that the final step in threonine biosynthesis in yeast required two enzyme fractions, Hser kinase (1) and a second fraction catalyzing an elimination of P_i coupled with the isomeration to form L-Thr from HserP (2).

$$\text{L-Hser} + \text{ATP} \longrightarrow \text{HserP} + \text{ADP} \quad (1)$$

$$\text{HserP} + H_2O \longrightarrow \text{L-Thr} + P_i \quad (2)$$

$$\text{L-Hser} + \text{ATP} + H_2O \longrightarrow \text{L-Thr} + \text{ADP} + P_i \quad (3)$$

The seemingly needless expenditure of phosphate bond energy has been visualized as a device to shift the equilibrium in favor of the accumulation of Thr [27]. According to this scheme HserP should only be an intermediate which once formed ought to disappear. It has not so far been possible to show the occurrence of reaction (1) in extracts of *Neurospora* [27]. Taking all this in consideration the reason for the presence of the comparatively large amounts of HserP in the TCA extracts of *L. casei* is not clear. Meanwhile it can be stated that by following the method described an alternative and easy way is given to obtain considerable amounts of highly labelled HserP. The labelling will be determined by the amount of radioactive inorganic phosphate added to the medium.

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