

BEHAVIOUR OF CARP PROTEINS AND CORN PROTEINS IN THREE-PHASE PARTITIONING

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In three-phase partitioning (TPP) ammonium sulphate and 2-methyl-2-propanol (*tert*-butanol) are used for separation of proteins by collecting and concentrating them into the middle layer or third phase of the system. During the last two decades, TPP has been primarily applied to enzyme purification at laboratory scale, however, its analytical type application for investigating meat has also been reported. Here, an experimental set of TPP feasible to analytical scale experiments is described to study partitioning behaviour of carp and corn proteins.

In partitioning experiments, corn proteins extractable with borate buffer, and sarcoplasmic proteins of carp were investigated. In the pH-range of 4.8–7.9, composition of partitioned corn proteins showed independence of pH as it was indicated by nonequilibrium pH gradient electrophoresis (NEpHGE) patterns. In case of carp, the alterations of protein composition in the pH-range of 3.7–8.0 unambiguously indicated the pH-dependent partitioning of carp proteins.

The analytical scale method, based on three-phase partitioning is a new approach to investigate food proteins.

Keywords: three-phase partitioning, carp sarcoplasmic proteins, corn proteins

Three-phase partitioning (TPP) is a widely used enrichment method for purification of proteins and other macromolecules. The basis of TPP is that 2-methyl-2-propanol forms a separated phase on 20% relative saturation (0.82 M) or more concentrated ammonium sulphate solutions. The method is essentially salting-out of proteins with ammonium sulphate in the presence of organic solvent 2-methyl-2-propanol (ODEGAARD et al., 1984; DENNISON & LOVRIEN, 1997; SHARMA & GUPTA, 2001). Common use of the salt and organic solvent leads to precipitation of proteins at the interface, where a so-called third phase is formed. The partitioning is influenced by pH, temperature and composition of the system, as well as by isoelectric point, molecular mass, hydrophobicity, solubility and concentration of the protein. The association of 2-methyl-2-propanol with the proteins (PIKE & DENNISON, 1989) governs the formation and position of the third phase. The third phase can be compressed by centrifugation, and using control samples and centrifugation at different speeds, information can be obtained for origin of proteins, of which the third phase is composed (SZAMOS et al., 1998).

TPP is a quick, efficient and inexpensive protein purification step that leaves impurities in the aqueous and organic phases. Owing to advantageous features of the

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method, we used TPP for purification of horseradish peroxidase (SZAMOS & HOSCHKE, 1992) and that of DNA (JÁNOSI & SZAMOS, 2001).

The pH of the aqueous phase of the three-phase systems plays a central role in enzyme isolation experiments, however, the pH dependence of three-phase partitioning of multi-component food protein mixtures has not yet been investigated.

The aim of present work was to develop an analytical scale three-phase partitioning method to study partitioning behaviours of animal and plant proteins. For elaboration of TPP at small scale, carp sarcoplasmic proteins and total soluble proteins of corn were used. TPP of carp and corn proteins was carried out in a system with low denaturing power, which consisted of small amount of ammonium-sulphate and 2-methyl-2-propanol. The protein composition of third phases was analysed by nonequilibrium pH gradient electrophoresis (NEpHGE).

1. Materials and methods

1.1. Materials

Ammonium sulphate was of analytical grade (Merck) and 2-methyl-2-propanol was of HPLC-grade (Sigma). All reagents for electrophoresis were of analytical grade or higher, other reagents were of analytical grade.

Prepared, deep-frozen Tükrös carp samples were obtained from Attala Fish Ltd, Attala, Hungary.

Corn cultivars Borbála and Gazda were obtained from Boly Agricultural Co., (Boly, Hungary) and NK 643 variety was from Monsanto.

1.2. Methods

1.2.1. Isolation of total soluble proteins of corn. Corn grains were ground to flours, which were completely sieved through 500 µm mesh sieve and used for experiments. Corn proteins were isolated according to the method of Wallace (HAMAKER et al., 1995). Flours were extracted with borate buffer (0.0125 M Na-borate, 1% sodium dodecyl sulphate, SDS, 0.5% 1,4-dithiothreitol, DTT, pH 10) in 1:10 ratio for 1 h on a shaker machine (Assistent, Karl Hecht Ag) at room temperature. The suspension obtained was centrifuged at 5000×g and 20 °C for 20 min. Supernatant was collected and stored as aliquots at -25 °C.



Fig. 1. Plastic separator for isolation of spun drip

1.2.2. Isolation of carp sarcoplasmic proteins. Three slices per fish were cut out of white muscles of thawed carps longer than 15 cm (A.O.A.C., 1996). With a cork borer like tool, 0.5–1.0 cm thick discs of 2 cm diameter were cut out of muscle slices. Discs were pierced at the centre and put onto a plastic separator, which was constructed considering the results on spun drip of PENNY (1975), and those of KOCHER and FOEGEDING (1993) on determination of water holding of protein gels. Devices containing muscle discs were put into 50 ml Falcon tubes and centrifuged at $650\times g$ at 15 °C for 20 min (Fig. 1). Spun drips obtained from the same fish were combined and stored as aliquots at –25 °C.

1.2.3. Three-phase partitioning of corn and carp proteins. The preparation of three-phase systems in 2 ml plastic tubes was carried out in the same sequence, as illustrated in Table 1.

Saturated salt solution, distilled water or buffer, protein sample solution and 2-methyl-2-propanol were successively added in order to prepare 21/30 systems [21% relative saturation (0.86 M) ammonium sulphate, 30% (v/v) 2-methyl-2-propanol, respectively]. The systems were incubated at 25 °C and then shaken 15 times up and down, and were let to stand for 15 min. The first centrifugation was at $200\times g$, using a swing-out rotor of Sigma 1–13 microcentrifuge (B. Braun) for 1 min. The thickness of the third phase was measured (B value in cm), then centrifugation was repeated at $4500\times g$ for 1 min, and the thickness of compressed third layer was measured again (C value in cm).

Table 1. Composition of three-phase systems used for investigation of carp and corn proteins

pH	Saturated ammonium sulfate (ml)	Buffer (ml)	Distilled water (ml)	Protein solution (ml)	2-Methyl-2-propanol (ml)
3.7	0.21	0.2 acetate 0.1 M, pH 3.6	0.54	Carp sarcoplasmic 0.05	0.43
4.4	0.21	0.2 acetate 0.1 M, pH 4.6	0.54	Carp sarcoplasmic 0.05	0.43
5.5	0.21	—	0.74	Carp sarcoplasmic 0.05	0.43
8.0	0.21	0.06 1M Tris-HCl pH 8.8	0.68	Carp sarcoplasmic 0.05	0.43
4.8	0.21	0.44 acetate 0.1 M, pH 3.6	—	Corn 0.35	0.43
6.7	0.21	0.44 acetate 0.1 M, pH 4.6	—	Corn 0.35	0.43
7.9	0.21	—	0.44	Corn 0.35	0.43

1.2.4. Nonequilibrium pH gradient electrophoresis (NEpHGE) in slab format. NEpHGE was carried out in the vertical cell of a Hoefer miniVE electrophoresis unit. Composition of 8 ml gel solution was: 4.4 g urea, 1.05 ml 30% (5.4% C) acrylamide solution, 0.44 g 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 0.4 ml Pharmalyte (pH 3-10), 3.34 ml distilled water, 10 µl N,N,N',N'-tetramethylethylenediamine (TEMED). After degassing the gel solution, 16 µl of 10% ammonium persulphate was added to it and was let to polymerise one night.

Electrophoretic conditions were: 400 V, 150 min.

Proteins in gel were detected by Coomassie G 250 colloid staining (NEUHOFF et al., 1988).

2. Results and discussion

Behaviour of carp and corn proteins in three-phase partitioning was examined on the basis of centrifuged three phase partitioning patterns, as well as electrophoretic protein patterns of third phases.

Data on three-phase partitioning patterns of carp proteins are illustrated in Table 2.

If C-values (layer thickness following centrifugation at 4500×g) are plotted against pH in the range of 3.7–8.0, the equation of linear regression is obtained:

$$C = 0.12 \text{ pH} - 0.36 \text{ (} r=0.95 \text{)}$$

In other words, third phases of carp sarcoplasmic proteins become thicker and less compressible (as it is indicated by R values) if partitioning occurs at higher pH values.

Interestingly, the electrophoretic pattern of carp proteins indicates a reduction of the protein content of the third phase at higher pH values, as can be seen in Fig. 2.

Table 2. Partitioning of carp proteins at various pH values in 21/30 systems

Sample	Aqueous phase pH	B (cm)	C (cm)	R= B/C
50 µl spun drip	3.7	0.44	0.1	4.4
50 µl spun drip	4.4	0.58	0.1	5.8
50 µl spun drip	5.5	0.95	0.4	2.4
50 µl spun drip	8.0	1.03	0.6	1.7

B: the thickness of the third phase following centrifugation at 200×g

C: the thickness of the third phase following centrifugation at 4500×g

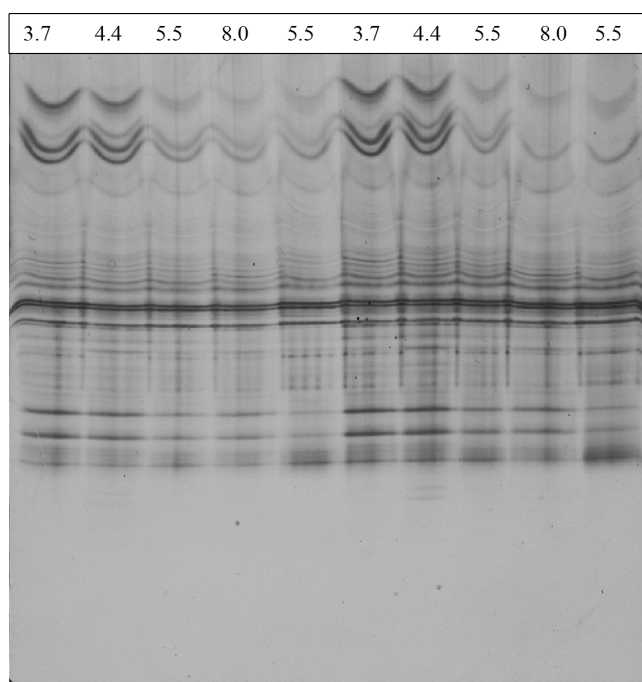


Fig. 2. NEpHGE-separation of carp sarcoplasmic proteins partitioned at various pH values (indicated in the row)

An increase of pH from 4.4 to 5.5 causes an increased dissolution of parvalbumins ['smiling' bands with low R_f (relative front) values] in the aqueous phase, that is, these proteins hardly participate in the formation of third phase above pH 5.5. This phenomenon can also be observed at the basic end of the gel, where proteins of high R_f tend to dissolve in aqueous phase at higher pH values (lanes 5.5 and 8.0), though to a much smaller extent. Partitioning of groups of proteins in the middle R_f -range (strong bands) is not influenced by changing pH. The partitioning behaviour of carp proteins can be exploited for separation of protein groups in one partitioning step. Thus, for

example, three-phase partitioning of carp proteins at pH 8.0 results in separation of parvalbumins from other sarcoplasmic proteins.

Behaviour of corn proteins in three-phase partitioning is characterized by data of Table 3.

Table 3. Partitioning of corn proteins in three-phase systems at various pH values

Corn cultivars	pH of partitioning	Amount of proteins (mg)	C (cm) at 4500×g	Amount of proteins in aqueous phase (mg)
Borbála	4.8	4.4	0.05	0.12
Borbála	6.7	4.4	0.03	0.11
Borbála	7.9	4.4	0.05	0.18
Gazda	4.8	4.3	0.05	0.02
Gazda	6.7	4.3	0.04	0.06
Gazda	7.9	4.3	0.03	0.08
NK 643	4.8	4.2	0.06	0.00
NK 643	6.7	4.2	0.04	0.09
NK 643	7.9	4.2	0.17	0.09

C: the thickness of the third phase following centrifugation at 4500×g

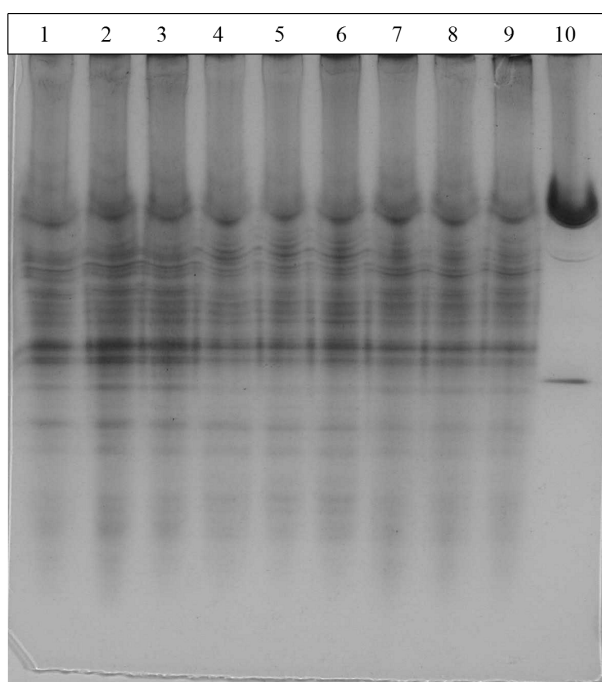


Fig. 3. NEpHGE-separation of corn soluble proteins partitioned at various pH values (Lanes 1, 2, 3: pH 4.8, 6.7, 7.9; Borbála, lanes 4, 5, 6: pH 4.8, 6.7, 7.9; Gazda, lanes 7, 8, 9: pH 4.8, 6.7, 7.9; NK 643, lane 10 marker protein, loading 10 µl/well)

As can be seen, C-values are very small and no correlation exists with pH of aqueous phases. The explanation of this behaviour is the low protein content of corn sample solutions (12 mg ml^{-1}) and the presence of alcohol-soluble zeins, which are dissolved in the 2-methyl-2-propanol phase and the involvement of which is limited to the formation of the third phase. In this case, the third phase is only composed of a fraction of the sample proteins, thus not representing the total soluble corn proteins.

As illustrated in Fig. 3, the protein composition of third phases does not show any alteration with increasing pH of aqueous phase in case of corn proteins.

Contrary to carp protein partitioning and on the basis of data obtained, corn protein partitioning is pH-independent in the range investigated, and separation of phase-forming protein groups is not feasible by alteration of pH of aqueous phase.

3. Conclusions

Summing up the results obtained, carp sarcoplasmic proteins and soluble corn proteins show distinct behaviour in three-phase partitioning systems of low denaturing effect, when the pH of the aqueous phases is changed.

From three-phase partitioning and electrophoretic patterns, it can be concluded that pH-dependence of carp proteins in TPP is a result of several processes: partly the amount of proteins in the third phase decreases, as the pH of the aqueous phase increases, partly the layer thickness C increases, as the pH increases in the range of 3.7–8.0. It can be stated that three-phase partitioning of carp sarcoplasmic proteins is pH-dependent in the range investigated.

On the contrary, behaviour of soluble corn proteins is pH-independent in the range investigated, inasmuch as three-phase partitioning patterns and electrophoretic patterns show no alteration with a change of pH.

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