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ELECTROPHORETIC STUDY OF POTATO TUBER PHOSPHOHYDROLASES

Our previous electrophoretic studies (Jaaska and Jaaska, 1968a, b) revealed that etiolated wheat and maize seedlings contained complex systems of phosphohydrolases consisting of several electrophoretically distinct fractions, some of which differed in substrate specificity and pH optimum. These studies also demonstrated that the electrophoretic patterns of soluble phosphohydrolases of maize seedlings were easily distinguishable from those of wheat seedlings by the electrophoretic mobilities of individual fractions. This evidence indicates the occurrence of species-specific differences in phosphohydrolase systems of the two plant species and suggests that phosphohydrolases of different plant species may be quite unlike in respect to their molecular structure and biochemical properties. For this reason, further comparative investigation of phosphohydrolase systems in different plant species is necessary for evaluating the degree of diversity and the most general properties of plant phosphohydrolases.

The present report extends our previous investigations of plant phosphohydrolases and deals with the results of polyacrylamide gel electrophoretic study of soluble phosphohydrolases, esterases, and proteins in potato tubers. Study of potato tuber phosphohydrolases has been the subject of many investigations since the work of Pfankuch in 1936 (Pfankuch, 1936). The properties of highly purified potato tuber phosphomonoesterase (Helferich, Stetter, 1947; Lora-Tamayo, Alvarez, 1959) and apyrase (Krishnan, 1949a, b; Molnar, Lorand, 1961; Liebecq *et al.*, 1963; Vernon *et al.*, 1965) preparations have been studied in detail. However, the number of different phosphohydrolases present in potato tubers and their substrate specificity still remain open.

Materials and Methods

Protein extracts were prepared from the tubers of white potato (*Solanum tuberosum* L., variety "Odenwald") and were subjected to vertical electrophoresis in polyacrylamide gel tubes. The zones of phosphohydrolase activity were visualized with the use of modified histochemical procedures. The methods used in this study were essentially those described in detail in our previous publication (Jaaska and Jaaska, 1968b). In addition, the triethylamine-molybdate method suggested by Sugino and Miyoshy (1964) for the specific precipitation of inorganic orthophosphate in the presence of various organic phosphates and inorganic pyrophosphate has been applied to visualize the sites of phosphohydrolase activity in polyacrylamide gels after incubation in the ordinary

Gomori's metal-salt reaction mixtures. Protein fractions were stained in a 0.2 per cent solution of Naphthol Blue-Black B(C. I. 20470) in a mixture of methanol-water-glycerine-acetic acid (5:5:1:1 by volume). The excess of dye was removed by washing in repeated changes of 5 per cent acetic acid.

Results

Fig. 1 presents polyacrylamide gel electrophoretic patterns of soluble phosphohydrolases from potato tubers revealed in acid medium at pH 5.2 or 6 by using different phosphate compounds as substrates. For comparison, electrophoretic patterns of soluble esterases active at pH 6 and of soluble proteins were also presented.

It is seen in Fig. 1 that at least 4—5 electrophoretically distinct fractions of varying staining intensity can be detected in the zymograms of ATPase and ADPase. The zymogram of 5′-nucleotidase revealed a broad intensely stained fraction and another less distinct one near the site of application of protein sample. The other zymograms obtained with the use of thiamine pyrophosphate (TPP), β-glycerophosphate, glucose-1-phosphate, and α-naphthyl phosphate as substrates each revealed one broad intensely stained fraction of coinciding electrophoretic mobility.

When comparing the zymograms obtained with the use of different substrates, characteristic differences in the substrate specificity of individual fractions can be seen. The zones of phosphohydrolase activity were numbered 1 through 5 in the order of decreasing mobility towards the anode. The most mobile phosphohydrolase corresponding to band 1 was able to split ATP and ADP, but revealed no detectable activity towards the phosphomonoester substrates used. Only when much longer incubation times were applied than ordinarily, a weak zone coinciding with band 1 appeared on the zymogram of TPPase. Thus, the enzyme(s) corresponding to zone 1 behaved essentially as an ATP-diphosphohydrolase or apyrase (EC 3.6.1.5) showing no phosphomonoesterase and only a small TPPase acivity. In most of the original zymograms it was possible to observe that zone 1 actually consists of two sub-bands which had close electrophoretic mobilities and were fused together in photographs.

Another major zone labelled 2 showed a broad substrate specificity hydrolyzing all the phosphoanhydride as well as phosphomonoester substrates tested. Zone 2 has been demonstrated with the use of both Gomori's lead-sulphide and triethylamine-molybdate methods of staining. Consequently, this enzyme can be classified as a nonspecific acid phosphohydrolase capable of splitting both phosphomonoester and phosphoanhydride bonds. Fraction 2 is seen in the zymograms as a single diffuse band. Actually it consists of three or four closely arranged sub-bands which were clearly distinguishable in most of the original zymograms, but were fused in photographs. The appearance of sub-bands suggests that potato tubers contain a system of isoenzymes of nonspecific acid phosphohydrolase.

Bands 3 and 4 revealed a weak activity towards ATP and ADP, but no detectable activity towards TPP, AMP and phosphomonoester substrates. Consequently, these enzymes can be classified as apyrases. The staining intensity of bands 3 and 4 was much weaker than that of band 1. The ATPase activity of fraction 3 is so weak that it is not reproduced in Fig. 1. In most of the original zymograms, however, band 3 is seen on both ATPase and ADPase zymograms.

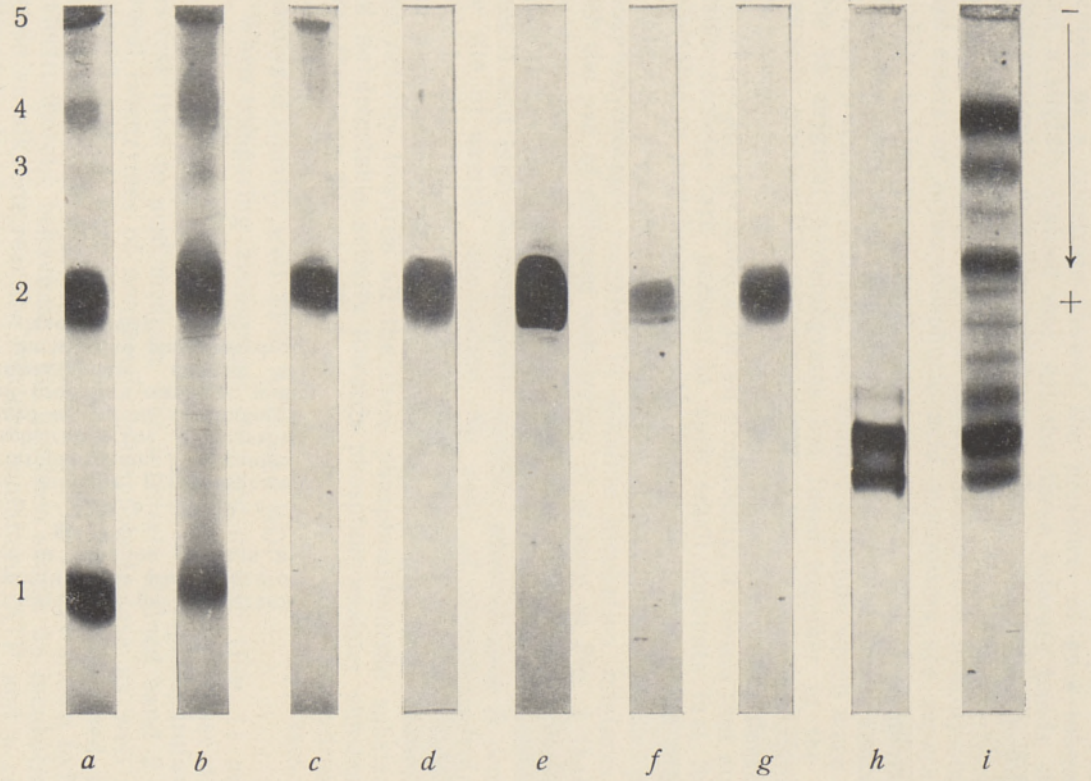

Fig. 1. Polyacrylamide gel patterns of soluble potato tuber phosphohydrolases, esterases and proteins.

Substrates: *a* — ATP, *b* — ADP, *c* — AMP, *d* — thiamine pyrophosphate, *e* — β-glycerophosphate, *f* — glucose-l-phosphate, *g* — α-naphthyl phosphate, *h* — α-naphthyl acetate, *i* — protein pattern. Zymograms *a* through *f* were obtained by means of Gomori's lead-sulphide method at pH 5.2, and zymograms *g* and *h* — by means of azo-dye staining method at pH of about 6.

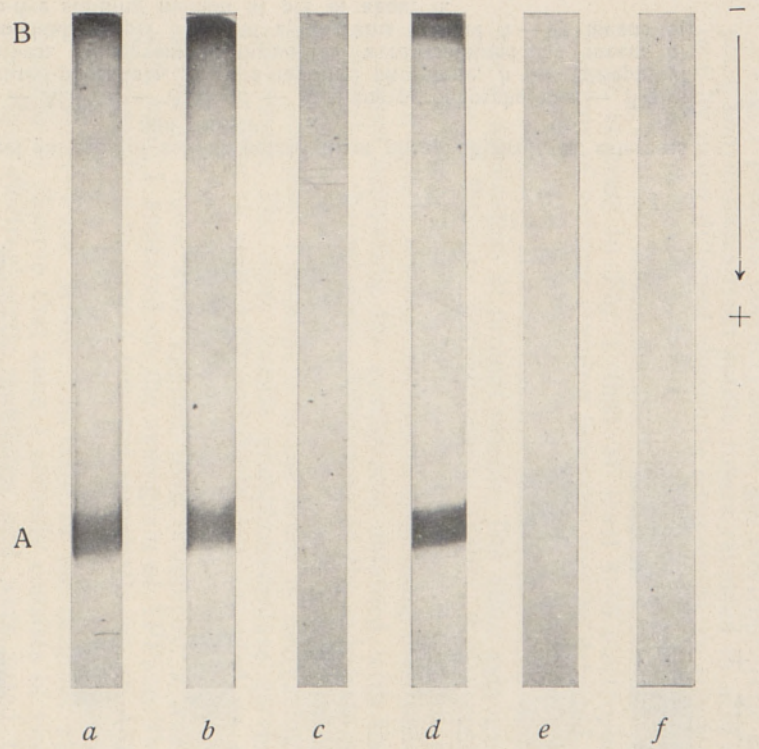

Fig. 2. Polyacrylamide gel zymograms of soluble potato tuber phosphohydrolases active in alkaline medium at pH 9.2.

Substrates: *a* — ATP, *b* — ADP, *c* — AMP, *d* — thiamine pyrophosphate, *e* — β-glycerophosphate, *f* — control with no substrate. The zymograms were obtained by means of Gomori's calcium salt procedure combined with triethylaminemolybdate method of visualizing the sites of phosphohydrolase activity.

Band 5 had only a slight electrophoretic mobility, remaining near the site of the application of the protein sample. It splits ATP, ADP, and AMP, but not TPP and phosphomonoester substrates. It can be suggested that fraction 5 consists of a mixture of phosphohydrolases with apyrase and 5′-nucleotidase activity which are particle-bound or poorly soluble in the buffer mixture used, and, therefore, had negligible electrophoretic mobility.

The zymogram of esterase (Fig. 1 *h*) obtained at pH 6 with α-naphthyl acetate as a substrate showed the presence of two intense fractions and a slightly stained one. All the three esterase bands showed close electrophoretic mobility which was, however, distinctly different from that of the nonspecific acid phosphohydrolase (band 2). It means that, at least in the case of potato tuber, the enzymes hydrolyzing carboxylic acid esters or ortho-phosphoric acid monoesters are separate proteins.

Fig. 1 also presents the polyacrylamide gel electrophoretic pattern of soluble proteins of potato tuber which revealed the presence of at least 11 main fractions. Actually, the number of bands which were distinguishable in original proteinograms but fused together in the photograph was even greater. When comparing the phosphohydrolase zymograms and the proteinogram, it can be seen that no protein bands developed at the site of the apyrase band 1. In all probability, it means that enzymatically active proteins are present in such small amounts that they are not detectable in proteinograms with the use of conventional protein stains.

Fig. 2 presents polyacrylamide gel electrophoretic patterns of phosphohydrolases revealed in alkaline incubation mixtures at pH 9.2 with the use of different substrates. It is seen in Fig. 2 that at pH 9.2 the zymograms of ATPase, ADPase, and TPPase all revealed identical patterns having intensely stained zones which were designated *A* and *B*. Zone *A* had high electrophoretic mobility whereas zone *B* remained near the site of sample application. No zone of phosphohydrolase activity could be detected on zymograms when phosphomonoester substrates AMP and β-glycerophosphate were used. Accordingly, both enzymes revealed high activity towards all the three phosphoanhydride substrates tested, and no detectable activity towards phosphomonoester substrates. Therefore, they can be specified as nonspecific phosphoanhydride phosphohydrolases. The enzymes cannot be classified as apyrases since they hydrolyzed equally well TPP, ATP, and ADP.

When comparing zymograms obtained in acid (Fig. 1) and alkaline (Fig. 2) media, substantial differences in phosphohydrolase patterns can be noted. In alkaline medium the number of phosphohydrolase fractions was less than in acid medium. The intensely stained band 2 comprising several isoenzymes of nonspecific phosphohydrolase as well as apyrase bands 3 and 4 were absent on the zymograms obtained in alkaline medium. Band 1 in Fig. 1 and band *A* in Fig. 2 were shown to be identical in electrophoretic behaviour. This has been shown by incubating the gels first in an alkaline reaction mixture containing ATP and a calcium salt followed by incubation in an acid reaction mixture containing ATP and a lead salt. It permits to reveal both "alkaline" and "acid" phosphohydrolases in the same gel and to compare more precisely their electrophoretic mobilities. Thus, potato phosphohydrolases corresponding to bands 1 (Fig. 1) and *A* (Fig. 2) in alkaline medium hydrolyzed actively all the three phosphoanhydride substrates used and behaved, therefore, as nonspecific phosphoanhydride phosphohydrolases, whereas in acid medium they revealed high apyrase activity with only traces of TPPase activity.

Slow-moving phosphohydrolase bands 5 (Fig. 1) and *B* (Fig. 2), not migrating far from the sample slot, also differed in respect of their substrate specificity. Band *B* showed high activity towards TPP in alkaline medium, whereas band 5 revealed no detectable TPPase activity in acid medium.

Discussion

The data of polyacrylamide gel electrophoretic study presented in this paper revealed, in potato tubers, the presence of three main types of phosphohydrolases which can be classified as non-specific acid phosphohydrolases, ATP-diphosphohydrolases (or apyrases) and non-specific phosphoanhydride phosphohydrolases. The same three types of phosphohydrolases have been found previously (Jaaska, Jaaska, 1968b) in etiolated maize seedlings. Of these enzymes only ATP-diphosphohydrolase (EC 3.6.1.5) can be found in the List of Enzymes presented by the Commission on Enzymes of the International Union of Biochemistry (Dixon, Webb, 1964). Therefore, the inclusion of the other two types of phosphohydrolases in the List of Enzymes requires consideration.

Several investigators (Ito *et al.*, 1955, Mayer *et al.*, 1961; Nagai, Funahashi, 1962; Shaw, 1966; *etc.*) have separated, from different plant tissues, highly purified phosphohydrolase preparations which were capable of hydrolyzing both phosphomonoester and phosphoanhydride substrates. Our gel electrophoretic studies of phosphohydrolases from etiolated maize seedlings (Jaaska, Jaaska, 1968b) and from potato tubers support the evidence that plant tissues contain nonspecific phosphohydrolases hydrolyzing both phosphomonoesters and phosphoanhydrides. In the case of using Gomori's lead-sulphide staining method to reveal phosphohydrolases after electrophoretic separation, the possibility must be considered that nonspecific acid phosphomonoesterases may split not the terminal phosphoanhydride bonds but phosphomonoester bonds with the liberation of pyrophosphate which also precipitates lead ions. It has been, however, shown that in acid medium the same ATPase pattern can be demonstrated with the use of both lead-sulphide and triethylamine-molybdate staining methods, thus suggesting the release of ortho-phosphate ions due to phosphohydrolase activity. From the above observation it is apparent that we cannot classify the enzymes hydrolyzing both phosphomonoesters and phosphoanhydrides with the liberation of inorganic phosphate as phosphomonoester phosphohydrolases (EC 3.1.3.2) since they also split terminal phosphoanhydride bonds. It has been proposed to classify these enzymes as nonspecific (acid) phosphohydrolases.

Our data presented some evidence that potato tubers contain a system of isoenzymes of nonspecific acid phosphohydrolase possessing very close electrophoretic mobilities. Isoenzymes of nonspecific acid phosphatase have previously been found in rice ears (Ikawa *et al.*, 1964), in wheat germ (Brouillard, Ouellet, 1965) and in orange peels (Schormüller *et al.*, 1965). Brouillard and Ouellet (1965) revealed, in wheat germ, four isoenzymes of nonspecific phosphatase which also exhibited esterase activity splitting both p-nitrophenyl acetate and p-nitrophenyl phosphate. Isoenzymes of potato tuber nonspecific acid phosphohydrolase (band 2 in Fig. 1), however, showed no activity towards α-naphthyl acetate as a substrate. Esterases and nonspecific acid phosphohydrolases found in potato tuber revealed clearly distinct electrophoretic mobility suggesting that in this tissue the two activities belong to separate proteins.

Another type of phosphohydrolases found previously in maize seedlings (Jaaska and Jaaska, 1968b) and now in potato tubers was specified as a nonspecific phosphoanhydride phosphohydrolase. This enzyme has been distinguished from ATP-diphosphohydrolase (apyrase) by its ability to hydrolyze actively TPP in addition to ATP and ADP, and from nonspecific phosphohydrolase by its inability to split phosphomonoester substrates. In potato tuber, two electrophoretically distinct fractions of nonspecific phosphoanhydride phosphohydrolase both active in alkaline reaction media have been demonstrated. One of the fractions (band *A* in Fig. 2) was shown to be identical in its electrophoretic mobility to an apyrase fraction on the zymogram obtained in acid medium (band 1 in Fig. 1). From the identity of electrophoretic mobility it is possible to assume that phosphohydrolase activity exhibited by the fraction in both acid and alkaline media may belong to the same enzyme (or enzymes, since fraction 1 was found to consist of two sub-bands). In this case the substrate specificity of the phosphohydrolase and even its classification seems to depend on the pH of the reaction medium since fraction 1 revealed in acid medium only a slight activity towards thiamine pyrophosphate compared with high activity towards this substrate in alkaline medium. In analogy with our results, Molnar and Lorand (1961) and Liébecq *et al.* (1963) found that the pH optima of purified potato apyrase preparations towards different phosphate substrates vary substantially. Therefore the possibility exists that the same phosphohydrolase may be classified differently depending on the conditions of enzymatic reaction. In our case, fraction 1 which was developed in acid medium was classified as an ATP-diphosphohydrolase, whereas fraction A of the same electrophoretic mobility, but revealed in alkaline medium, behaved as a nonspecific phosphoanhydride phosphohydrolase. It must be emphasized, however, that on the basis of identical electrophoretic mobility alone we cannot suggest certainly that the enzymatic activity belongs to a single protein and this possibility needs to be checked with the use of other protein fractionation methods.

At present it is difficult to distinguish clearly two types of potato phosphohydrolases — ATP-diphosphohydrolases and nonspecific phosphoanhydride phosphohydrolases. Potato apyrase has recently been shown (Liébecq *et al.*, 1962, 1963) to be not specific for ATP, but to split other nucleoside tri- and diphosphates also. For this reason it seems to be more justified to classify potato apyrase not as ATP-diphosphohydrolase but as nucleoside triphosphate-diphosphohydrolase, as it was suggested by Liébecq *et al.* (1962) and Hadjiolov (1964). By means of DEAE-cellulose chromatography Liébecq *et al.* (1962, 1963) have been able to separate, from a crude potato apyrase preparation, two fractions of different substrate specificity, which were specified as a nucleoside triphosphate diphosphohydrolase and nucleoside triphosphate phosphohydrolase. The last more specific type of potato phosphoanhydride phosphohydrolase has not been found under the conditions of our gel electrophoretic study. Partially purified potato apyrase preparation freed of phosphomonoesterase activity was shown by Cori *et al.* (1965) to split actively, besides ATP and ADP, also a synthetic non-nucleotidic phosphoanhydride substrate phenyl-propyl triphosphate. It is suggested here to specify the phosphohydrolases capable of hydrolyzing actively both nucleotidic and non-nucleotidic phosphoanhydride substrates without showing phosphomonoesterase activity as nonspecific phosphoanhydride phosphohydrolases.

Summary

Soluble phosphohydrolases, esterases, and proteins of potato tubers were studied by means of vertical polyacrylamide gel electrophoresis combined with histochemical methods of staining.

Three main types of phosphohydrolases have been found which were specified, on the basis of the substrate specificity, as nonspecific acid phosphohydrolases, ATP-diphosphohydrolases (apyrases), and nonspecific phosphoanhydride phosphohydrolases. In acid medium (pH 5.2), 3—4 closely moving isoenzymes of nonspecific acid phosphohydrolase and 4—5 fractions of ATP-diphosphohydrolase (apyrase) were detected by using different phosphate substrates. In alkaline medium (pH 9.2), two electrophoretically distinct fractions were found. Both fractions hydrolyzed ATP, ADP and thiamine pyrophosphate with no activity towards phosphomonoester substrates, and were specified as nonspecific phosphoanhydride phosphohydrolases.

The esterase zymogram with α-naphthyl acetate as a substrate revealed two intensely stained zones and a weak one. Potato tuber esterases and nonspecific acid phosphohydrolases were shown to have clearly different electrophoretic mobilities and, therefore, to be separate enzymes.

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KARTULIMUGULATE FOSFOHÜDROLAASIDE ELEKTROFOREETILINE UURIMINE

Resümee

Kartulimugulate lahustuvate fosfohüdrolaaside, esteraaside ja valkude fraktsioonilist koostist uuriti vertikaalse polüakrüülamiidgeelelektroforeesi meetodi abil.

Erinevaid fosfaatseid substraate kasutades täheldati happelises keskkonnas (pH 5,2) 3—4 lähedase elektroforeetilise liikuvusega mittespetsiifilise happelise fosfohüdrolaasi isofermenti ja 4—3 ATF-difosfohüdrolaasi (apüraasi) fraktsiooni. Leeliselises keskkonnas (pH 9,2) ilmnes ensümogrammidel ainult kaks erineva elektroforeetilise liikuvusega fosfohüdrolaasi, mis mõlemad toimisid fosfoanhüdriidsetele, mitte aga fosfomonoestrilistele substraatidele. Substraatse spetsiifilisuse alusel jaotati kartulimugulate lahustuvad fosfohüdrolaasid kolme põhirühma: 1) mittespetsiifilised happelised fosfohüdrolaasid, 2) ATF-difosfohüdrolaasid ja 3) mittespetsiifilised fosfoanhüdriidide fosfohüdrolaasid.

Esteraasi ensümogrammil (substraat — 1-naftüülatsetaat) esines üks madala ja kaks kõrge aktiivsusega tsooni, mis kõik tunduvalt erinesid mittespetsiifilise happelise fosfohüdrolaasi isofermentidest oma elektroforeetilise liikuvuse poolest. Proteinogrammil täheldati vähemalt 11 valgufraktsiooni.

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Saabus toimetusse
25. IV 1968

ВЕЛЛО ЯАСКА

ЭЛЕКТРОФОРЕТИЧЕСКОЕ ИЗУЧЕНИЕ ФОСФОГИДРОЛАЗ КЛУБНЕЙ КАРТОФЕЛЯ

Резюме

С помощью вертикального электрофореза в полиакриламидном геле в сочетании с гистохимическими методами окрашивания изучался фракционный состав растворимых фосфогидролаз, эстераз и белков клубней картофеля.

Выявлены три основных типа фосфогидролаз, которые на основании субстратной специфичности классифицировались как неспецифические кислые фосфогидролазы, АТФ-дифосфогидролазы (апиразы) и неспецифические фосфогидролазы фосфоангидридов. В кислой среде (pH 5,2) обнаружены 3—4 изофермента неспецифической фосфогидролазы, обладающие близкой электрофоретической подвижностью и расщепляющие как фосфоангидридные (АТФ, АДФ, тиаминпирофосфат), так и фосфомоноэфирные субстраты (2-глицерофосфат, АМФ, глюкозо-1-фосфат и 1-нафтилфосфат). Кроме того, выявлены 4—5 фракций АТФ-дифосфогидролазы, гидролизующие АТФ и АДФ, но не обладающие заметной фосфогидролазной активностью в отношении тиаминпирофосфата и фосфомоноэфирных субстратов. В щелочной среде (pH 9,2) выявлены лишь две фракции фосфогидролаз. Обе фракции активно расщепляли все три испытанных фосфоангидридных субстрата, не проявляли фосфомоноэстеразной активности и поэтому классифицировались как неспецифические фосфогидролазы фосфоангидридов.

На зимограмме эстеразы при использовании 1-нафтилацетата в качестве субстрата выявлены три фракции. По электрофоретической подвижности изоферменты эстеразы существенно отличались от изоферментов неспецифической кислой фосфогидролазы и, следовательно, являются различными белками. На протеинограмме обнаружено по крайней мере 11 основных фракций белков.

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