再現生機－由蝌蚪尾巴探討動物再生

獲獎榮譽榜

參加美國第五十二屆國際科技展覽會
獲
大會獎動物學科四等獎

作者：陸惠宗
就讀學校：高雄高中
指導老師：梁靖薇　洪志宇

「大處著眼，小處著手」，這次很高興有機會能夠將我大膽的構思，經過一步一步的小心求證，在多次嘗試錯誤中找出答案，而這件作品呈現在國際科展的選拔裡，獲得教授們的肯定，是對我最大的鼓勵！我覺得研究科學，「創意」是很重要的。從小我就對科學研究有濃厚的興趣，今年是高雄中學二年級，此次能獲選參加美國國際科展，感到很榮幸。感謝我的生物老師梁靖薇一路陪著我走過來，這當中我們經歷過從許多的挫折、失敗，到後來的成功、喜悅，這些都將是我人生中寶貴的經驗。出國期間，感謝多位教授的指導，特別是林榮耀教授讓我受益良多，也感謝家人的支持與祝福。

研究動機

動物的再生和發育是個非常複雜且有趣的議題。包括牛蛙在內的無尾類兩生類，在變態為成蛙的過程中，蝌蚪的尾巴均會消失。但是若切除蝌蚪的部分尾巴，則被切除部分會再生出新的片段來。這表示，在切除傷口表面下的表皮細胞會分泌出某種因子使表皮細胞去分化成為具有再生潛力的胚幹細胞。接著，細胞大量增殖，再分化以完成整個再生的過程。根據前人的研究，已知 retinoic acid 和 thyroxine 能夠影響已分化狀態的細胞之去分化。我想了解在再生過程一開始時，啟動分子是如何作用的，特別是蛋白質的差異表現。因此，我利用蛋白質 2 維電泳來分離再生尾巴的蛋白質。再進一步利用所得的等電點和分子量與網路上的蛋白質資料庫比對。

研究方法

A.生物材料：

從當地的水生養殖場取得 70 隻第 24 期，約 8cm 長的牛蛙蝌蚪，先將 70 隻蝌蚪分為實驗組（A 組），對照組（B 組）兩大組，各 35 隻。其中每 5 隻蝌蚪再分為 A1， A2，A3，...A7；B1， B2， B3...B7。在實驗開始的第零天，切除所有實驗組（A 組）蝌蚪的尾巴末端 1cm，但是作為對照用的 B 組則不切尾巴。隔天（實驗第一天），取 A1 組的蝌蚪，再切取 1cm 的尾巴保存以便後續的分析。B1 組則切取與 A1 相對的 1cm 尾巴，保存以作為對照組用。重複上述的取樣步驟，直到所有組別（A2~A7；B2~B7）都取樣完成。

B.2 維電泳技術：

1.取樣而得的尾巴樣本，直接以液態氮冷藏保存，接著再將之冷凍乾燥，磨碎後，儲存在 -70℃備用。將 1 毫克的粉狀樣本溶解在 200 μ l 的萃取緩衝液中，接著以 BCC 法測定其蛋白質濃度。將蛋白質萃取液進行 2 維電泳，作進一步的分析。

2.由 -20℃冷凍冰箱中取出 2 維電泳專用的 strip 解凍，然後泡在事先配好的平衡液中 12 個小時。開啟冷卻循環設備使 2 維電泳平台保持在 15℃，接著把 2 維電泳平台上鋪上一層煤油以便導電，最後再放上電泳槽。將泡好平衡液的 strip 取出，放在溼濾紙上，吸去多餘的平衡液後即可放入電泳槽中。將專用的導電棉紙分別扣緊在 strip 的兩端，再把 loading 樣本用的 cups 扣入 strip 的前端，並將電泳槽注滿矽油。將所要進一步研究的蛋白質萃取液和 marker loading 到 cups 中，接上電源供應器先以 300V 、 0.5mA 、 5W 處理一個小時，再以 3500V 、 0.5mA 、 5W 處理 18 個小時。將經過第一次 2 維電泳處理的 6 條 strip 浸泡在甘油中，放入 -75℃冷凍設備中保存備用。先取出 loading 有 A1 、 B1 蛋白質的兩條 strip ，分別浸泡在平衡液 A 及平衡液 B 中， 15 分鐘後取出，放在濕濾紙上，吸去多餘的平衡液。將 2 維膠版平鋪在 2 維電泳平台上，開啟冷卻循環系統，使平台保持在 15℃，接著在膠版兩端分別放上正、負極的導電膠。把浸過平衡液的 strip 以及 marker loading 到 2 維膠版上，開啟電源供應器，先以 600V 、 30mA 、 20W 處理 30 分鐘，再以 600V 、 50mA 、 30W 處理 70 分鐘。將跑完電泳的膠版取出，立刻用甲醇和乙酸的混合固定液固定其上的蛋白質，然後再依步驟進行硝酸銀染色，以便觀察。

3.將所得的組織蛋白質圖譜以電腦掃瞄，以便於在 NCBI 網站（http://www.ncbi.nlm.mih.gov）和 ExPASY 網站（http://www.expasy.ch）搜尋相關期刊論文與已知蛋白質片段的條件，以了解此段蛋白質的功能。

結果

在切除尾巴的實驗組中，再生速率在 3 、 4 、 6 天分別出現了二個高峰期。在對照組中第 5 天則有較快的再生速率。但在實驗組中的第 7 天及對照組的第 6 天，尾巴長度均發生了減縮的情形。

經由蛋白質 2 維電泳，發現了有二個蛋白質在再生的過程中發生了明顯的變化。接著再把包含這二個蛋白質的區域 Fig3-I ， Fig3-II 放大成為 Fig3-III ， Fig3-IV ，被標定為 P1 的蛋白質（分子量 10000 道耳吞；等電點 5.7 ）其總量明顯地在所有的再生實驗組別中增加。另一個被標定為 P 2 的蛋白質（分子量 14000 道耳吞，等電點 7.5 ）則出現在實驗組中的第 3 、 4 、 6 天（再生速率較快）以及全部的對照組別中。

討論

經由在網路上資料庫的搜尋，發現 P1 擁有與 ICAT 相同的蛋白質特徵，而 ICAT 被認定為能夠透過抑制 T-cell factor 和 beta-catenin 之間的交互作用機制來負調節 Wnt signaling 。因此， P1 可能和發育與細胞增生有關，而在組織再生中扮演著相當重要的角色。

P2 穩定地存在於所有的對照組已分化細胞中，但是在網路上的蛋白質資料庫中卻無法發現。 P2 蛋白質可能具有維持已分化細胞停留在分化完成的狀態。在實驗組中，已分化的細胞在尾巴被切除後即重返細胞生命循環，故當這些細胞處於去分化階段時（ A1 ， A2 ， A5 ）， P2 蛋白質即消失，但是當再分化作用完成後， P2 又會再出現了。

結 論

1.在整個蝌蚪尾巴組織再生過程中，發現了兩個蛋白質具有相當顯著的變化，其一被初步鑑定為ICAT，另一個仍未知，卻可初步預測其可能具有維持已分化細胞停留在已分化狀態的功能。

2.利用蛋白質2維電泳來追蹤觀察在尾巴組織中的蛋白質是個相當實用的方法，進而能比較那些有差異變化的蛋白質，而能預測其功能為何。當然，更深入的氨基酸定序工作則能夠對於我們所要的目標蛋白質再獲得更準確的辨識資料。

參考資料

1.Ben Herbert, Advances in protein solubilisation for two-dimensional electrophoresis,Electrophoresis,20(1999).

2.Donald C. Lo, Francesca Allen, and Jeremy P. Brockes, Reversal of muscle differentiation during urodele limb regeneration, Proc. Nal. Acad. Sci. USA, 90(1993)

3.Jeremy P. Brockes, Amiphibian limb regeneration: rebuilding a complex structure, Science, 276(1997).

4.Jiri Stulik, Kamlia Koupilova, Lenka Hernychova, Ales Macela, Vaclav Blaha, Claudia Baaske, Walter Kaffenberger, Dirk van Beuningen, Modulation of signal transduction pathways and global protein composition of macrophages by ionizing radiation, Electrophoresis, 20(1999).

請參照英文作品說明書後面所附的圖片，每個圖片的中文解說如下：

Fig.1.(在英文作品的P.2)

A.實驗組蝌蚪　　B.對照組蝌蚪

取樣部分　之前已切除部分　　取樣部分　非取樣部分

Fig.2.(在英文作品的P.9的後面)

蝌蚪尾巴每日的生長長度，這顯示尾巴的成長速率

Fig.3. 以2維電泳分析由蝌蚪尾巴所分離出來的蛋白質

I. A組

II. B組

III. A組的局部放大

IV. B組的局部放大

Search for Protein(s) Differentially Expressed During Tissue Regeneration of the Tadpole tail

Introduction

The development and regeneration of animals are very complicated and interesting issues. In Anura amphibians, including Rana catesbeiana (bullfrog), tails of tadpoles disappear during transformation into frogs. Those tails can also regenerate after amputation. It was demonstrated that the epithelium under the wound would produce an active factor which will cause the dedifferentiation of the epithelium into blastema. Subsequently, the cells proliferate, differentiate, and complete the regeneration process (Brockes, 1997). Previously, it was shown that retinoic acid and thyroxine can influence the reversal of the differentiated state (Lo et al., 1993). I was interested in the earliest molecular event(s) that happens in the regeneration process, especially the changes of protein expression in the regenerating tails. Therefore, I utilized two-dimensional gel electrophoresis (2-DE) to analyze the proteins in the regenerating tadpole tail. The characteristics, isoelectric point (pI) and molecule weight (Mw), of the differentially expressed proteins were compared to those in the database on Internet (NCBI: www.ncbi.nlm.nih.gov ; ExPASy: www.expasy.org).

Materials and Methods

Materials

Bullfrog tadpoles (stage 24, approximately 8 cm in length) were obtained from a local aquaculture farm. For each experiment, 70 tadpoles were evenly divided into two groups, the experimental group A and the control group B. Every five tadpoles, for subgrouping, were designated as A1,A2……A7; B1, B2……B7. In the beginning of the experiment (Day 0), 1 cm of the distal tail was removed from each tadpole in groups A (including A1 to A7) while those in groups B were remained untouched. On the next day (Day 1), 1 cm was removed from the regenerating tails in subgroup A1, while the corresponding segments were collected from subgroup B1 and used as the controls. The cutting steps were repeated every day in the rest of experimental subgroups, A2 to A7, and control subgroups, B2 to B7.

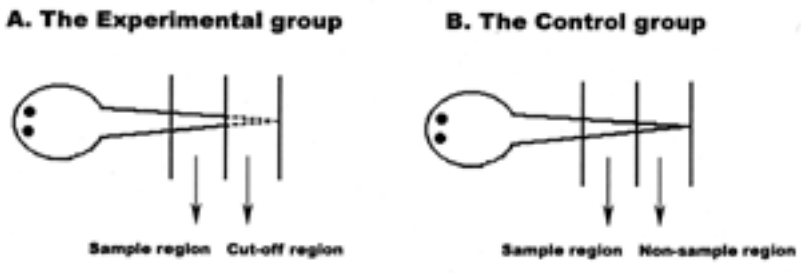


Fig.1 Sample region

Sample preparation for electrophoresis

The samples obtained from the operation were directly frozen in liquid nitrogen, lyophilized, pulverized, and stored at –70°C until use.

Approximately 1 mg of the powdered sample was dissolved in 200µl lysis buffer composed of 8 M urea, 4 % CHAPS, 40 mM Tris , 65 mM DTE (Add shortly before use), and a trace of bromophenol blue. The protein concentrations were then determined by the Coomassie brilliant blue method (CBB).

Two-dimensional gel electrophoresis (2-DE)

Samples (60µg of protein) were separated by 2-DE, which was performed by using immobilized pH gels for the first dimension and sodium dodecyl sulfate (SDS) gels for the second dimension. Prior to isoelectric focusing (IEF), the immobilized pH gel strips were rehydrated, and then run in an IPGphor horizontal electrophoresis apparatus (Pharmacia-Biotech, Uppsala, Sweden). The experiment was conducted at 200 V for 30 min, and continued at a linear-gradient voltage setting initially at 300 V and increased gradually until 3500 V (final voltage) for a total of 5 kVhr. Immediately after IEF, the gels were equilibrated with 100 ml of equilibrated buffer containing 2 % 1,4-dithioerythritol at room temperature for 15 min and then transferred onto the top of SDS gels, and electrophoresis were performed first at 400 V and 40 mA for 10 min, and then at 400 V and 80 mA for 4 hr. The proteins in gels were visualized by silver staining, and the stained gels were scanned and analyzed with the Malanie 3 software (GeneBio S. A.).

Results

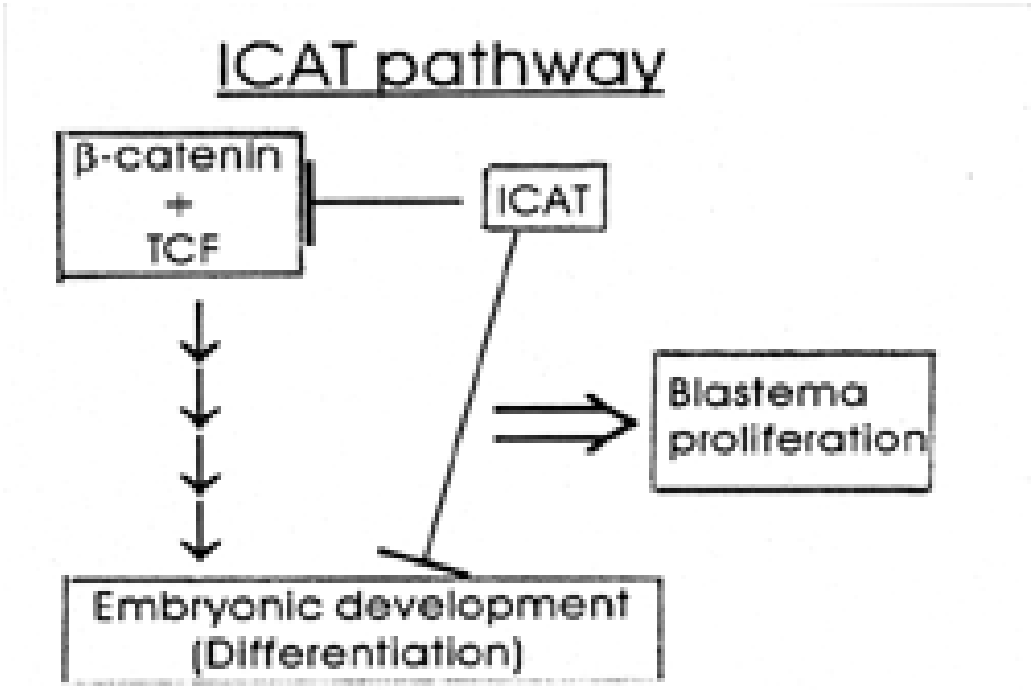
There are two peaks of regeneration rate in tail-amputated groups at third to fourth, and sixth day, respectively (Fig.3). In control groups, the fifth day had the higher regeneration rate. Both in the seventh day of the tail-amputated groups and sixth day of the control groups, the tail length was reduced.

By analysis of 2-DE, it was found that there were two proteins significantly changed along the regeneration situation (Fig.4-I, Fig 4-II). By the enlargement of the area containing the two proteins, in Fig. 4-I, Fig. 4-II, it was revealed that in Fig. 4-III, Fig. 4-IV, the protein designated as P1 has a molecular mass of ten kDa and pI of 5.7, and that the amounts of P1 were remarkably increased in all the regenerating tails (in tail-amputated groups A1 to A6) and in the tails under rapid growth (control groups B5 to B6). Furthermore, another protein designated as P2 has a molecular mass of fourteen kDa and pI of 7.5 which appeared at the third, fourth, and sixth day (with faster regenerating rate) in the tail-amputated groups, and in all of the control groups.

Discussion

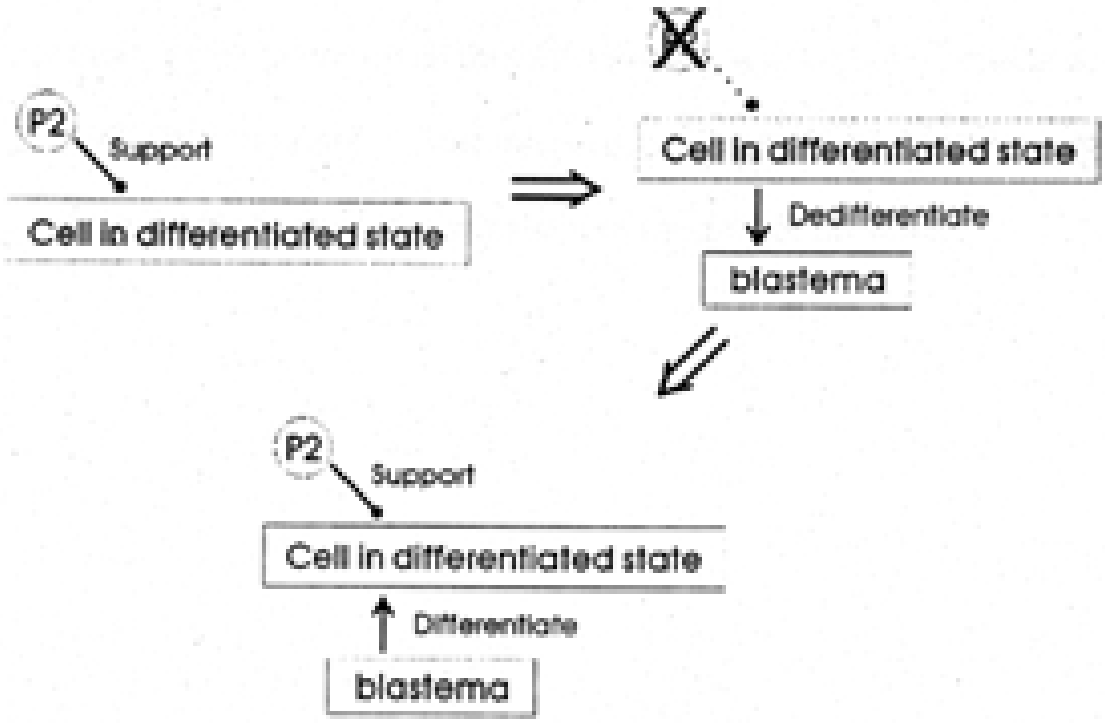
By searching the protein database on Internet, it was found that P1 has the same characteristic as those of beta-catenin-interacting protein (ICAT). Its function is considered negatively regulating Wnt signaling via inhibition of the interaction between beta-catenin and T-cell factor (TCF). Therefore, P1 could be involved in development and cell proliferation, and play significant role(s) in tissue regeneration.

Expression of ICAT					
Group	A1	+	Group	B1	-
Group	A2	+	Group	B2	-
Group	A3	+	Group	B3	-
Group	A4	+	Group	B4	-
Group	A5	+	Group	B5	-
Group	A6	+	Group	B6	-



P2 could not be identified by protein database, which was steadily found in the differentiated cells in the control groups. Protein P2 could play a role of supporting cells in the differentiated state as shown in the control groups B1 to B6. In the tail-amputated groups, the differentiated cells reentered into the cell cycle after amputation. During dedifferentiation (tail-amputated groups A1, A2, A5), protein P2 disappeared but reappeared at the stage when differentiation was complete (tail-amputated group A6).

Expression of ICAT					
Group	A1	-	Group	B1	+
Group	A2	-	Group	B2	+
Group	A3	+	Group	B3	+
Group	A4	+	Group	B4	+
Group	A5	-	Group	B5	+
Group	A6	+	Group	B6	+



Conclusions

1.Two proteins were found with marked alterations in the expression patterns in the tadpole tail tissue during the regeneration process. One is putatively identified as ICAT; the other is a novel protein P2 with a predictive supporting function.

2. Utilizing 2-DE to monitor the proteins inside the tail tissue is a practical method, even to compare the differentially expressed proteins and to predict what they are. Furthermore, amino acid sequencing could be performed for further identification of the target proteins.

References

1.Ben Herbert, *Advances in protein solubilisation for two-dimensional electrophoresis, Electrophoresis, 20* (1999).

2.Donald C. Lo, Francesca Allen, and Jeremy P. Brockes, *Reversal of muscle differentiation during urodele limb regeneration, Proc. Nal. Acad. Sci. USA, 90* (1993).

3.Jeremy P. Brockes, *Amiphibian limb regeneration:rebuilding a complex structure, Science, 276* (1997).

4.Jiri Stulik, Kamlia Koupilova, Lenka Hernychova, Ales Macela, Vaclav Blaha, Claudia Baaske, Walter Kaffenberger, Dirk van Beuningen, *Modulation of signal transduction pathways and global protein composition of macrophages by ionizing radiation, Electrophoresis, 20* (1999).

Figures

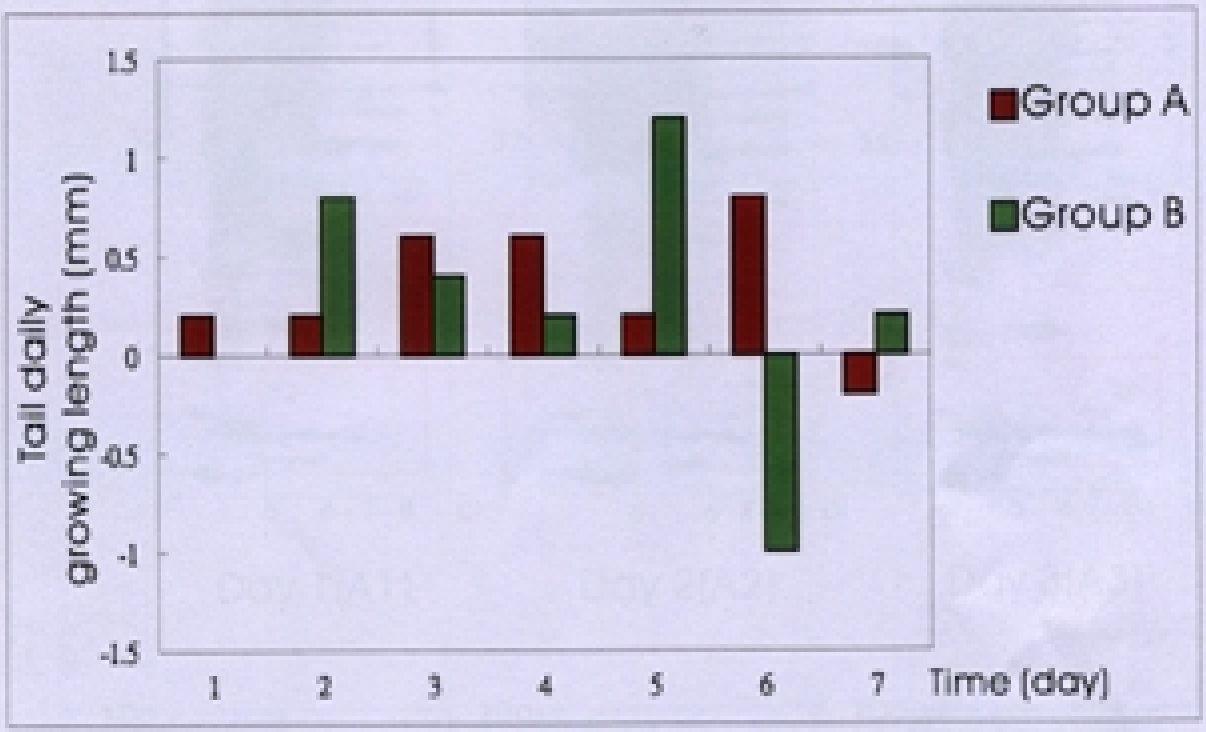
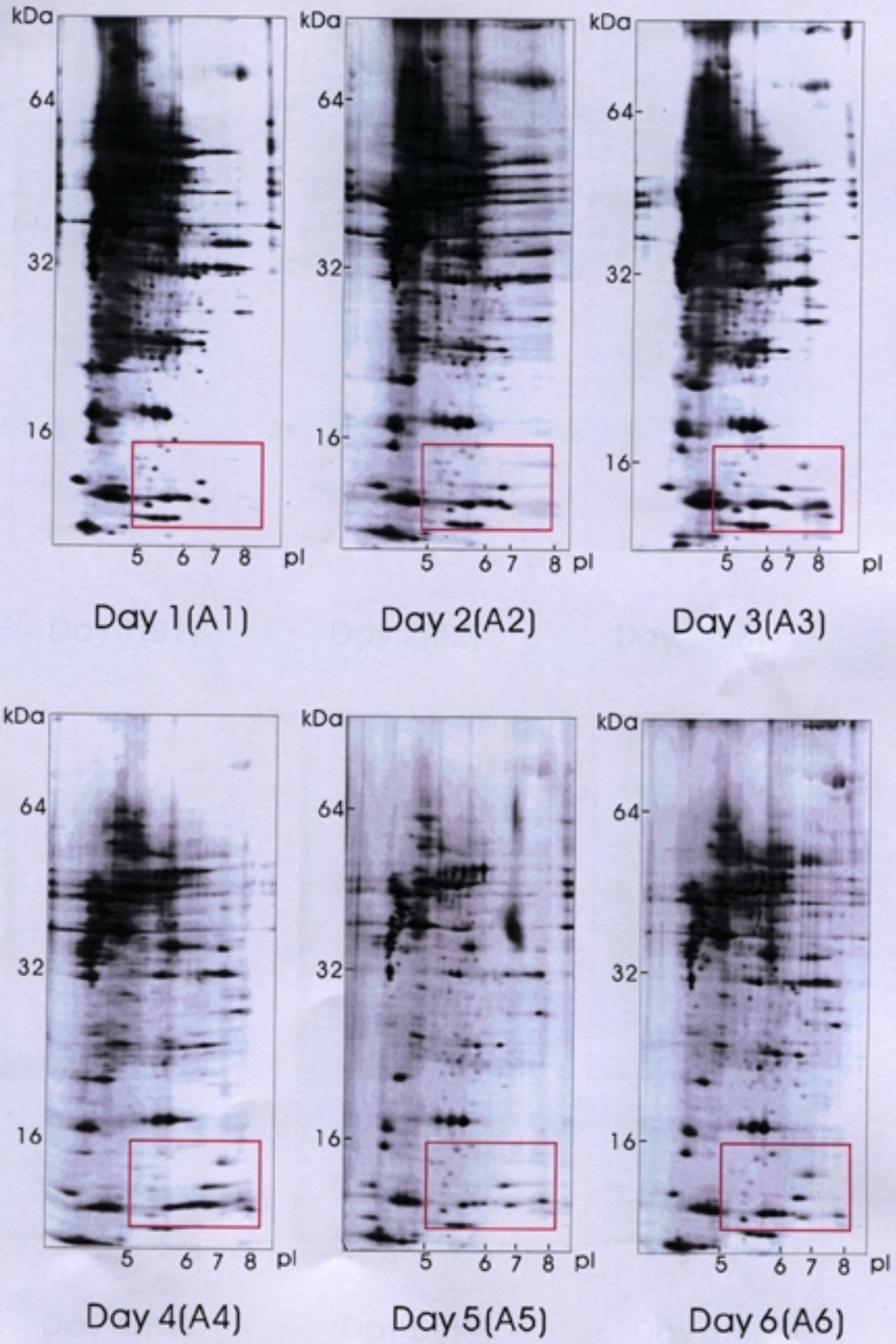


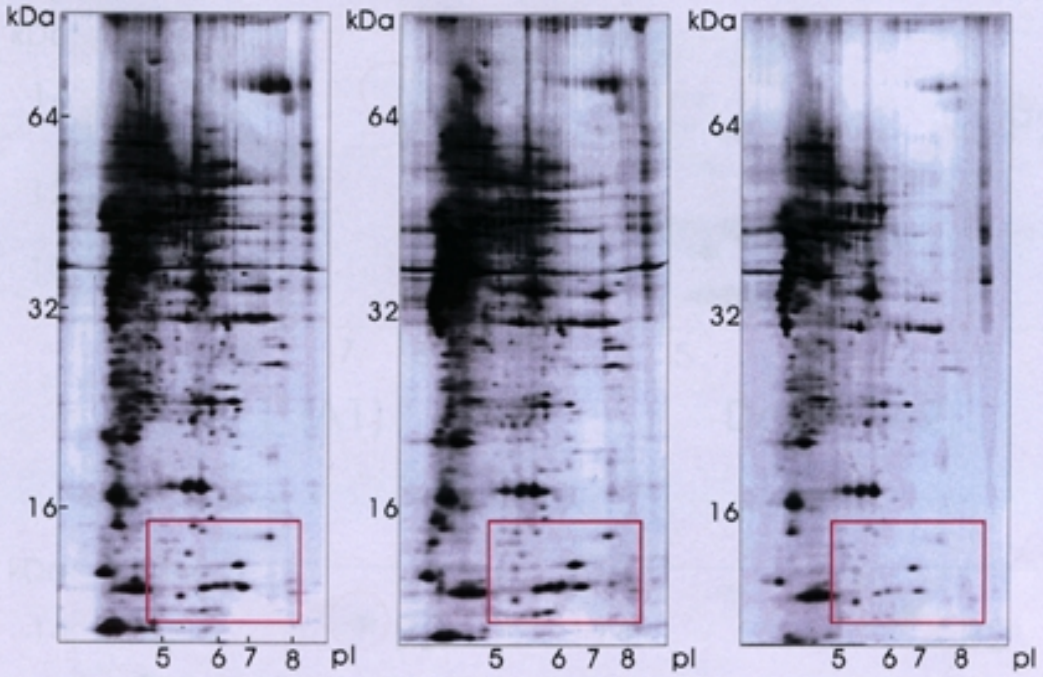
Fig.2 Daily growing length of the tadpole tails. It is shown that the rate of the tail growth.

Fig.3 2-DE analysis of proteins isolated from tadpole tails

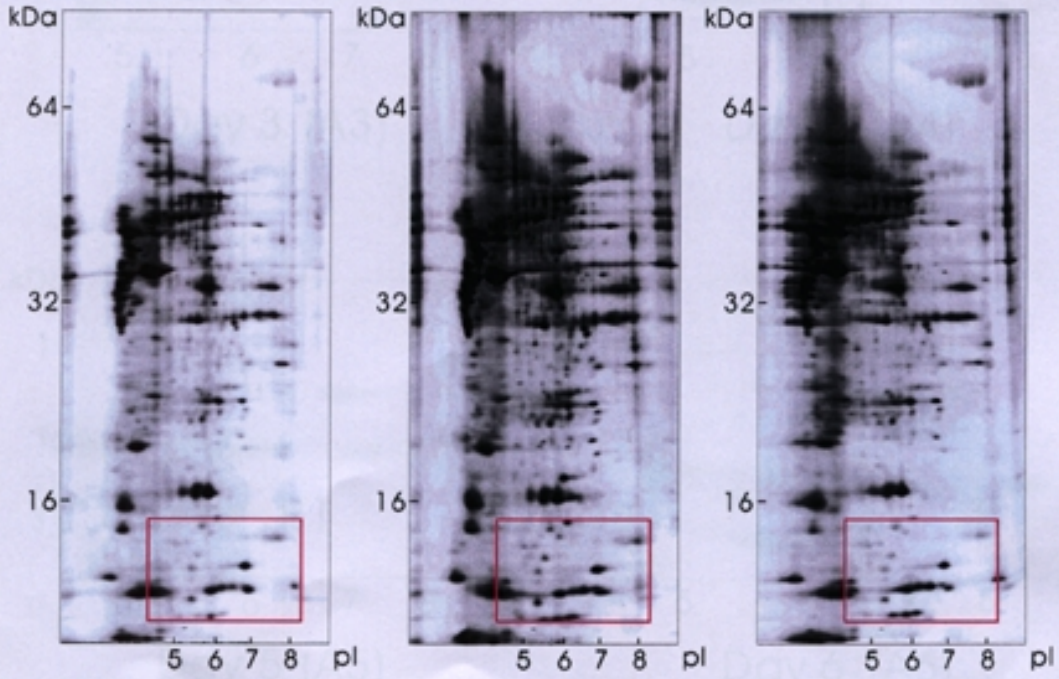
I. Groups A



II. Groups B

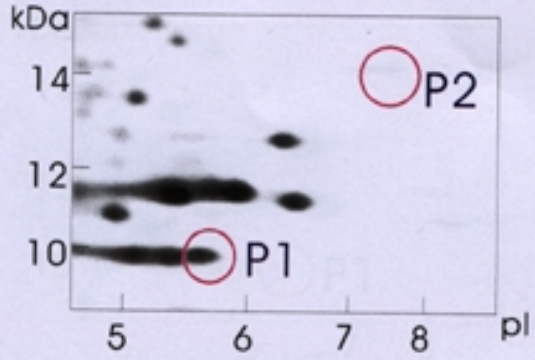


Day 1(B1) Day 2(B2) Day 3(B3)

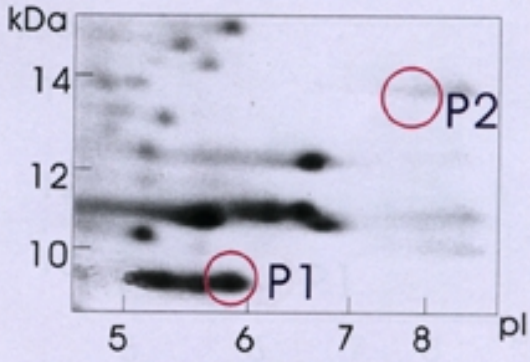


Day 4(B4) Day 5(B5) Day 6(B6)

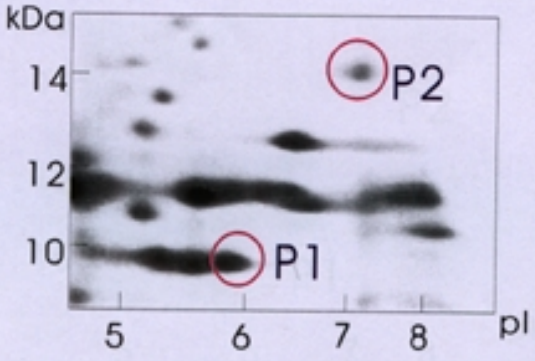
III. Enlarged images of groups A



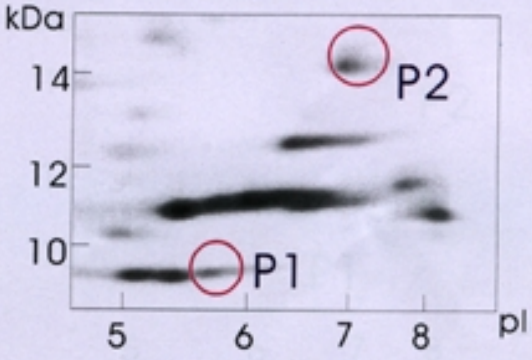
Day 1 (A1)



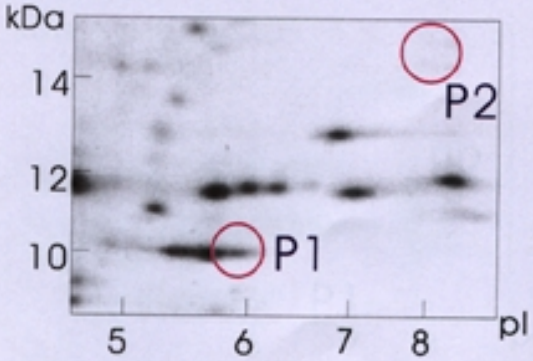
Day 2 (A2)



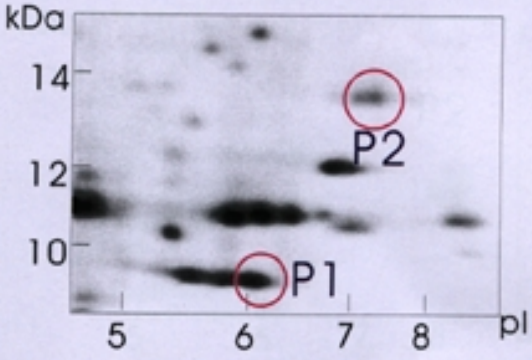
Day 3 (A3)



Day 4 (A4)

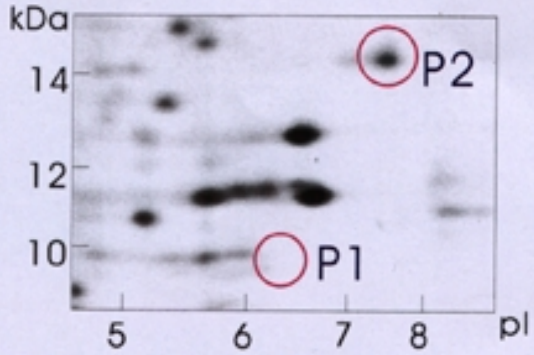


Day 5 (A5)

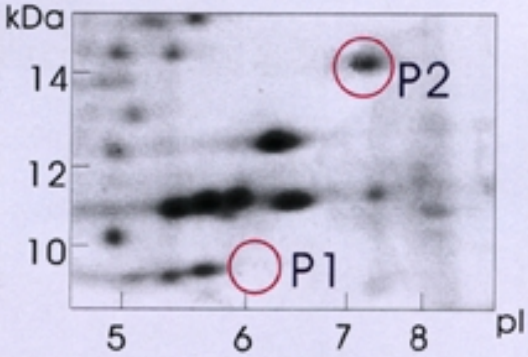


Day 6 (A6)

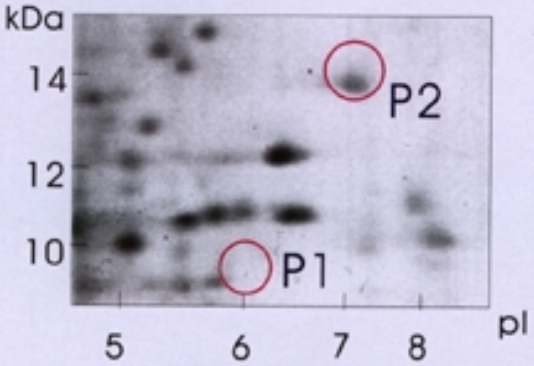
IV. Enlarged images of groups B



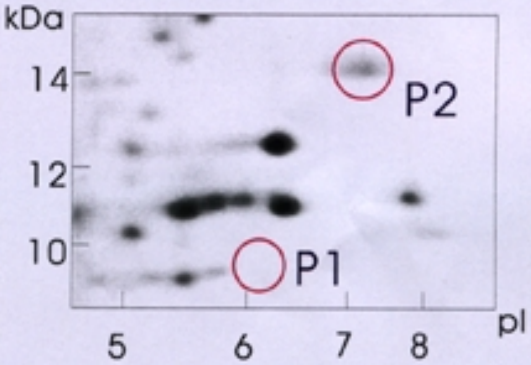
Day 1 (B1)



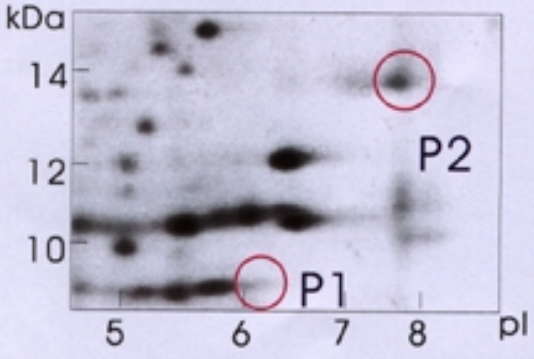
Day 2 (B2)



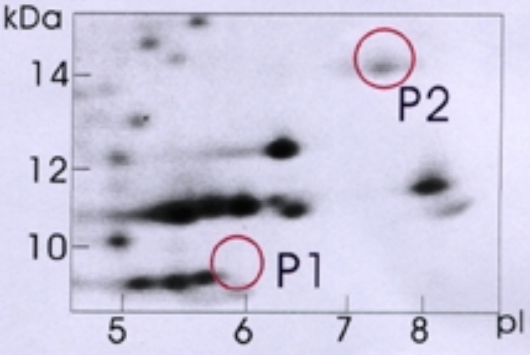
Day 3 (B3)



Day 4 (B4)



Day 5 (B5)



Day 6 (B6)