NOTEBOOK

June 2023

JUNE

HUBU-SKY-China

AUTHOR: Lijuan Hu

Breaking bacteria, purified (A2, T7, M8)

Thursday,June 1,2023

Overview

- Since we forgot to add PMFS on May 14, we continued the fragmentation and purification

Experimental equipment

① Broken bacteria

- 1 ×PBS buffer、 PMFS

② protein was purified

- Nickel beads, G250 Coomassie brilliant blue, and imidazole at each concentration

Experimental steps

①Broken bacteria

- 1 .1% PMFS was added to the bacterial solution, then the high pressure cell cruter was washed twice with RO water and 1 XPBS buffer (pressure controlled at 300bar)
- 2 . After washing the high pressure cell cruter, add the target solution for crushing (pressure controlled at 800bar~ 900bar)
- .3 According to the transparency of the broken bacteria liquid, break the bacteria for 3 to 6 times.
- .4 After the bacteria are broken, divide them with 2 m IEP tube, and leave the tube without centrifugation (sample preparation). After packing, centrifugation at 14000rpm, 10℃ for 30min.
- 5 . After centrifugation, keep- -tube precipitation, one tube supernatant (sample preparation). The remainder of the supernatant can be collected.

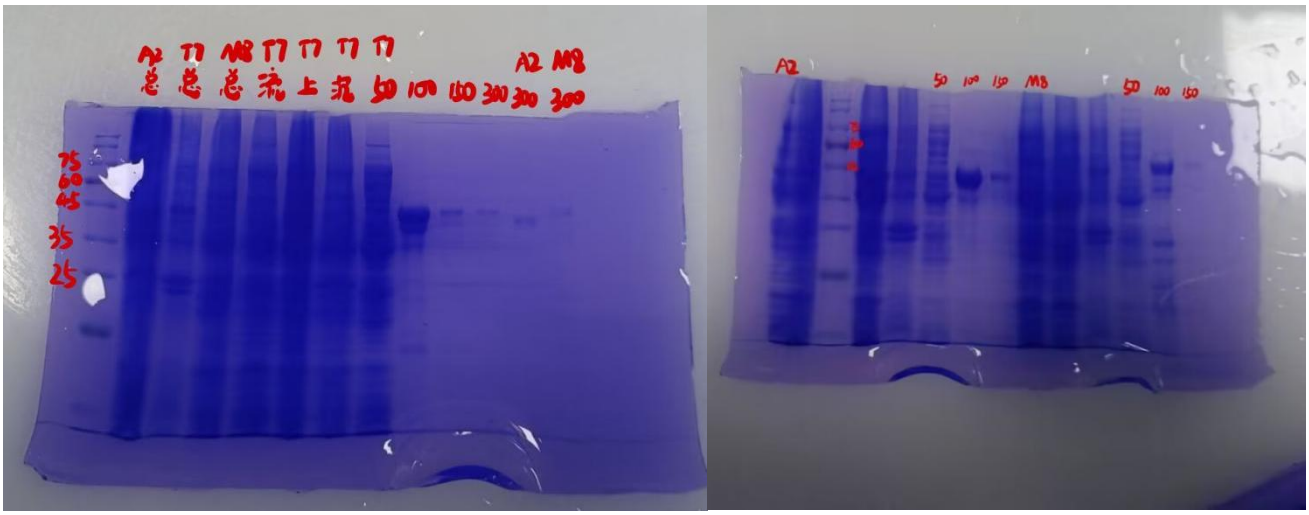
②Protein purification

- 6 . Appropriate amount of nickel beads was first added to the purified column and then washed twice with RO water and 1 PBS buffer.
- .7 After washing, the nickel beads were activated by adding 5min of 10 mM imidazole to the

purified beads supplemented with nickel beads.

- 8 . After activating the nickel beads, the collected supernatant night was added immediately, and the sealing tube mouth was mixed in the silent mixer for 60min (so that the target protein was fully bound to the nickel beads).
- 9 . After mixing, the fluid was collected and placed on ice.
- 10 Eution was performed using 10 mM imidazole, 30 mM imidazole, 50 mM imidazole, 100 mM imidazole, 150 mM imidazole, 300 mM imidazole, 600 mM imidazole. Each concentration of imidazole should be washed until the eluate meets G250 Coomassie blue.
- 11 . Sampling of precipitation, supernatant, fluid through, whole, and each concentration of imidazole eluate.40ul loading and 10u samples were mixed and boiled at 100℃ for 10min.
- 12 . After sample preparation, the target proteins were tested by SDS-PAGE. During loading, take 10ul of each sample and 2ul of protein marker.
- 13 . Superfiltration was performed according to the SDS-PAGE results.

Experimental results



ultrafiltration

Friday,June 2,2023

Overview

- The purified protein was processed by ultrafiltration to increase its concentration

Experimental equipment

- And 1 x pbs of the buffer
- The G250 Coomassie Bright Blue

Experimental steps

- 1. Pour the soaked RO water from the ultrafiltration tube, and add new RO water to the upper vessel, and leak at 4000rpm 10°C 1min in the centrifuge
- 2. After the leak detection, add the corresponding buffer to detect 4000rpm 10°C 1min in the centrifuge
- 3. After leak detection, the target protein solution is added, first 4000rpm 10°C 10min, take 10ul of upper and lower liquid respectively, and test with G250 (upper layer becomes blue and lower layer remains blue)
- 4. After G 250 test, pour the lower liquid and replenish the target protein liquid to the scale line and continue to centrifuge. 7. (twice)
- 5. Leave 500ul-1500ul of protein solution finally subpackaged (1.5ml EP tube after 200 ml of packaging, quick-frozen with liquid nitrogen and stored at-80℃)

Plasmids were extracted, concentration measured, nucleic acid dispensing, and transformed

Friday,June 22,2023

Overview

- .The E. coli plasmid containing ABCC, R6F-322, F7 8F, N23, A2, R6F3 genes was extracted by centrifugation, lyase and other methods
- The extracted plasmid will be measured for the DNA concentration
- The extracted plasmids were subjected to nucleic acid glue analysis
- The extracted plasmids were transferred into the competent bacteria

Experimental equipment

①Plasmids were extracted from

- E. coli bottles with different transcribed genes
- Pipette gun, gun head, centrifuge, 1.5ml， 2 . The omIEP tube
- Solution P1 (RNase A), P2, P3 rinse solution PW, adsorption column CP3 elution buffer EB

②The concentration was measured by

- Good extraction of different plasmids, EB solution
- Pipette gun, gun head
- NaNo Drop 8000 Measure the nucleic acid concentration meter

③ running nucleic acid glue

- Mix: 024g agarose + 30 ml TBE buffer
- Exextracted different plasmid solutions
- Nucleic acid glider
- Pipette gun, gun head, loading, 2000maker, 1kdmaker

④ change

- LB culture medium
- Pipette gun, gun head, alcohol lamp
- Antibiotics and ampicillin
- BL21-DE 3 competent cells (enabling protein expression)
- 1 .5mIEP tube

Experimental steps

①Plasmids were extracted from

- 1. Mark 72ml centrifuge tubes, pour different bacteria into the centrifuge tubes and centrifuge at 12000rpm for 1min, pour the supernatant, collect the bacteria. Repeat the operation until the centrifugation
- 2. Add 250ul of solution P1 (always add RNase A) to the centrifuge tube with the bacterial precipitate, and suspend the precipitate with a pipette
- 3. Add 250 ul of P 2 to the centrifuge tube, and gently flip it over 6-8 times to fully lyse the bacteria
- 4. Add 350ulP3 to the centrifuge tube, immediately gently turn up and down 6-7 times, fully mix, appear white precipitate, centrifuge at 12000rpm for 10min
- 5. Transfer the supernatant of the previous collection to the adsorption column cp 3 with a pipette (the adsorption column is placed in the collection tube). Pay attention not to remove the precipitate, centrifuge at 12000rpm for 1min, pour out the waste liquid in the collection tube, and put the adsorption column cp 3 into the collection tube
- 6. Add 600u rinse pw to the adsorption column cp 3, centrifuge at 12000rpm for 1min, pour out the waste liquid from the collection tube, and repeat this step once
- 7. The adsorption column cp 3 was put into a collection tube and centrifuged at 12,000 rpm for 2min to remove the residual rinsing solution in the adsorption column
- 8. Place the adsorption column cp 3 in a clean centrifuge tube, add 50ul of elution buffer EB to the middle of the adsorption membrane, centrifuge for 2 min12000rpm for 2min to collect the plasmid solution into the centrifuge tube, and mark it on the centrifuge tube

②Plasmid concentration was measured by

- 1. Wash the sample injection hole of the nucleic acid instrument with water and EB solution, respectively, and absorb the water and EB solution of the sample injection hole with absorbent paper
- 2. Draw 2-4ul of plasmid solution with a pipette gun and put it into the well
- 3. Cover the instrument lid, debug the equipment, and click to test
- 4. Record the concentration of different plasmids and take photos
- Supplement: The first three plasmid concentrations have multiple peaks, so we re-determined the first three samples, each sample in parallel peaks, the first peak, resulting in multiple

peaks; the single peak indicates that our extracted plasmid is relatively pure

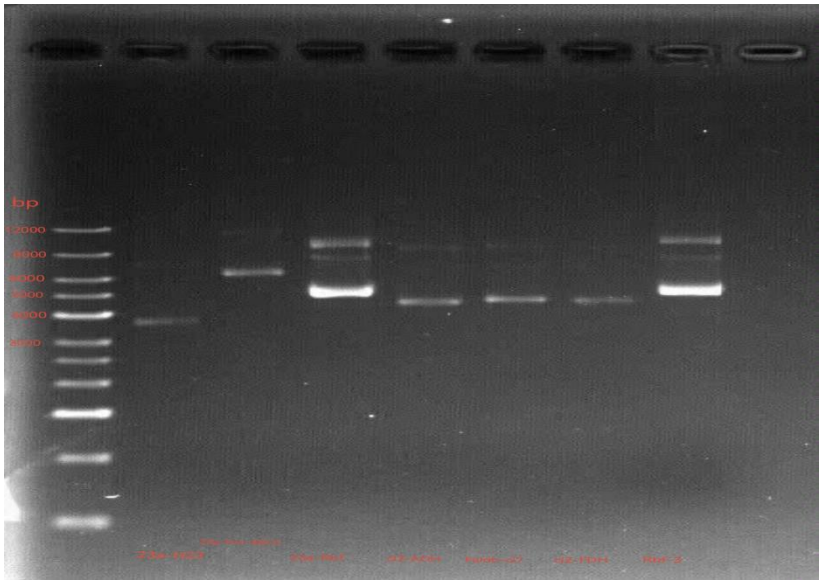
③ running nucleic acid glue

- 1. Mix: 024g agarose, 30 ml ETB buffer, microwave heating into a splint for 30min to make nucleic acid glue
- 2 .2ul of loading and 1u of different plasmid solutions were successively mixed evenly to the void, and 2u of 2000 and 1 kd maker were added to the void
- 3. Put the electrophoresis tank for electrophoresis 40-50min
- 4. Take out the rubber board and wash it carefully with water, then put it into the staining solution for 20min
- 5. Put the stained glue plate into the light glue instrument for observation
- Analysis: A small amount of the plasmid solution has multiple bands, possibly after a ring plasmid superhelix or break into a chain to cause the electrophoresis difference

④ change

- 1. 50ul 2000 kanamycin was added to LB medium to LK medium, 50ul 2000 ampicillin to LB medium and 50ul of 2000 kanamycin and ampicillin to LB medium
- 2. To 1 respectively in the ultra-net workbench.1 ml of different plasmids and 20 ml of competent cells were added to the 5-ml EP tube and marked on the tube accordingly
- 3. Heat shock: water bath in 42℃ water bath for 45s, and then immediately ice bath for 2min
- 4. 150ul of NZY medium was added to the different EP tubes on the ultra-clean workbench
- 5 This mixture was incubated at constant temperature in a 37℃ shaker for 50min
- 6. After 120ul of incubation, the bacterial solution was applied in LK, LB, double antibody plates, labeled and dated on the plate, and incubated in a 37℃ incubator overnight

Experimental results


bp
12000
8000
6000
5000
4000
3000

Pick bacteria

Saturday,June 23 ,2023

Overview

- The transformed medium was picked into a small bacterial liquid bottle to expand the culture

Experimental equipment

- Pipette gun, gun head
- Marker pen, alcohol bottle, workbench
- Small bacterial liquid bottle, LB medium
- Ampicillin, and antibiotics

Experimental steps

- 1. 50ul of ampicillin was added to LB medium, antibiotics and both to make the corresponding resistance medium
- 2. Pour the prepared resistance medium into the corresponding liquid bottle on the work table, no more than one third of the bacterial liquid bottle, and make the corresponding mark
- 3. Carefully pick up 3-4 single colonies with the head of the gun, move them into the corresponding small bacterial liquid bottle, and shake them well
- 4. Put the selected bacterial liquid bottle into a 37℃ shaker for overnight culture

Pick the bacteria again and turn the bottle

Saturday,June 24 ,2023

Overview

- The overnight culture results of the previous pick bacteria were not ideal, some bacteria in the bottle, and some have been precipitated, so repick bacteria to expand the culture
- The cultures grown in small bottles were transferred to large bottles of TB medium

Experimental equipment

① pick bacteria:

- Pipette gun, gun head
- Marker pen, workbench, alcohol bottle, alcohol lamp
- Small bacterial liquid bottle, LB medium
- Ampicillin, and antibiotics

② to bottle:

- Ampicillin, and antibiotics
- glycerol
- Centrifuge tube rack, 50ml centrifuge tube, 1.5mlEP tube, marker, alcohol lamp, alcohol bottle, workbench, pipette gun, gun head
- TB culture medium
- Buffer Buffer solution

Matters need attention

① pick bacteria:

- Mark the bottle body (date, corresponding to the name of the enzyme, the name of the resistance medium)
- Remove the flame of the bacterial liquid bottle after each move
- Pick 3-4 single colonies to make sure that there are bacteria every time

② to bottle:

- Mark the vial (date, the name of the enzyme, the name of the resistance medium)
- Shake the small liquid bottle

- Every time the bottle mouth and bottle seal the plastic film through the alcohol lamp flame

Experimental steps

① pick bacteria:

- 1. 50ul of ampicillin was added to LB medium, and the corresponding resistance medium was available
- 2. Pour the corresponding resistance medium into the small bacterial liquid bottle on the workbench, and the medium is not more than thirds of the small liquid bottle
- 3. Carefully pick up 3-4 single colonies with the head of the gun, move them into the corresponding small bacterial liquid bottle, and shake them well
- 4. in a 37℃ shaker for 6 – 8h

② to bottle:

- 1. Protect the bacteria: in 1. Write the name of the corresponding bacteria on the 5 m IEP tube, add 500U of glycerol and 500U in each tube, mix well, and keep it in an 80℃ refrigerator
- 2. Add 1L of TB medium, antibiotics, 500ul, 25ml of Buffer medium, half of the small bacterial solution bottle, and one bacterial solution to two bottles
- 3. Add the connected bacteria into 37℃ expanded culture for 5h with inducer

Turn the bottle again

Monday,June 26 ,2023

Overview

- When expanding the culture with the previous bottle, the inducer was neglected due to the induction time, and the bottle was continued to expand the culture

Experimental equipment

- Ampicyl hydropenicillin, antibiotics
- Pipe rack, 50ml centrifuge tube, marker, alcohol lamp, alcohol bottle, workbench, pipette gun, gun head
- 1 LTB medium
- phosphate buffer

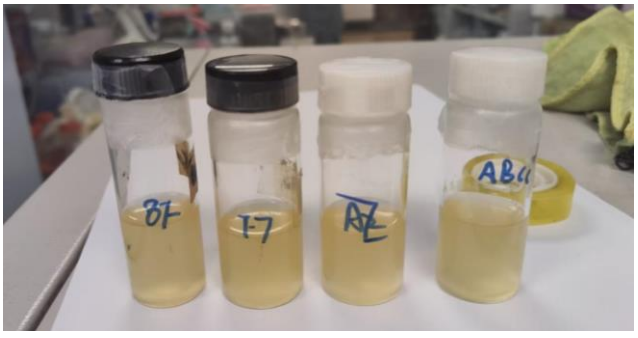
matters need attention:

- Mark the large bottle (date, the name of the enzyme, resistance medium)
- Shake the small liquid bottle
- Large bottles and sealing film remember the flame
- Do not reach into a large bottle when pouring bacteria, ampicillin, or antibiotics or buffers

Experimental steps

- 1. Before receiving bacteria, place the required items (except bacteria liquid) in the ultra-clean bench and ultraviolet irradiation for 10min
- 2. Add 4x 25ml of phosphate buffer, 2000x ampicillin 500ul or 2000 antibiotic 500ul or 500ul of each antibiotic to the TB medium
- 3. The bacterial TB medium was incubated in a 37℃ constant temperature shaker for 6-8h

Experimental results



Collect bacteria, prepare protein purification bacteria

Tuesday,June 27 ,2023

Overview

- 1. The large bottle of bacterial liquid was centrifuged yesterday
- 2. The N23 protein scaffold was not shaken up the day before yesterday, and the bacteria were picked up again

Experimental equipment

① receiving bacteria

- high-speed centrifuge
- receiving flask
- A 50-ml centrifuge tube
- glass rod

② Prepared for protein purification

- Purification column
- RO water

③ pick bacteria

- Pipette gun, small gun head
- superclean bench
- LA culture medium
- Bacterial bottle
- marker pen

matters need attention:

- 1 . When resuspended, the used glass rod should be cleaned to prevent bacterial contamination
- 2. Pick the colonies as gently as possible to avoid picking the medium

Experimental steps

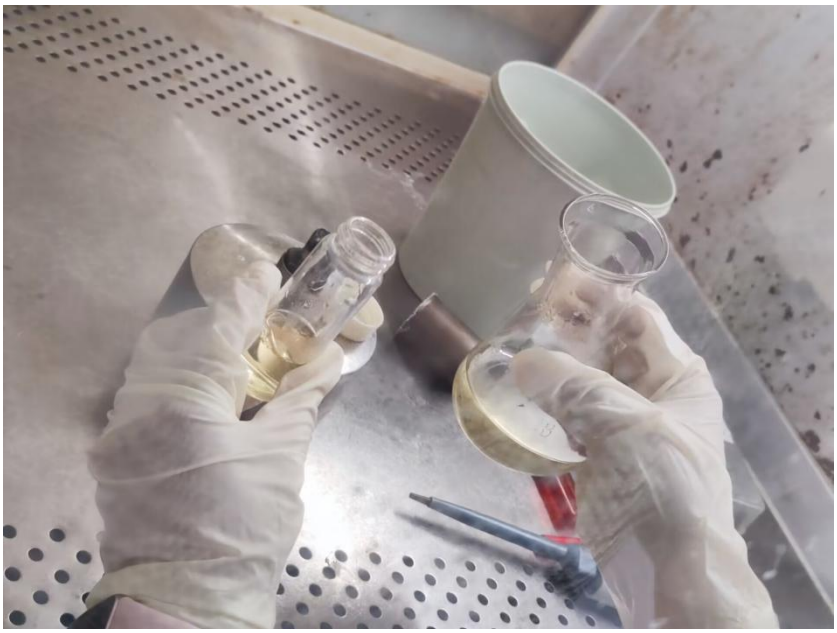
① receiving:

- 1 . The expanded culture of the shaker was loaded into the collection bottle and flattened in pairs and centrifuged at 6000 r/min for 10 minutes
- .2 The supernatant was inverted, resuspended with PBS buffer and transferred to a 50ml centrifuge tube in mated
- .3 was centrifuged at 6000 r/min 4℃ for 15min
- 4. Pour out the supernatant and keep it in the-20℃ refrigerator
- .5 Clean the ultrafiltration tube

③ pick bacteria:

- 6 . Pour 1 / 3LA medium into the bacterial vial on the-clean bench
- 7. Pick three single colonies with a small gun, move into a bacterial flask, and culture overnight

Experimental results



Ttered, purified, and-in gel

Wednesday,June 28 ,2023

Overview

- Bacteria containing the A2, F7 gene were fragmented to flow the corresponding protein t of the cell
- The proteins of the broken bacteria were affinity adsorbed with nickel beads and eluted with different concentrations of imidazole
- The eluted proteins were subjected to electrophoresis and analyzed

Experimental equipment

① broken bacteria

- Low temperature and high pressure breaker
- Pure water, and buffer in phosphate buffer
- 2mlEP tube
- A2, and the F7 bacterial solution

② purification

- Nickel beads, imidazole solutions at different concentrations
- Elution tube, 50ml centrifuge tube, 10mlEP tube, pipette gun, gun head

- Protease inhibitor pmfs, G250 Coomassie blue dye solution, 0.1M Nao H solution

③ running protein glue

- Protein solution with different concentrations of imidazole, 10 – 18kdmaker, loading
- Pipette gun, gun head
- Protein glue, with an electrophoresis apparatus

Matters need attention

① broken bacteria

- The pressure of cleaning is different from that and should be adjusted in time
- Clean the sample tube in exchange for a new bacterial liquid to be broken

② purification

- Samreaction with G250 at each elout
- Each eluent was filled with the corresponding protease inhibitor pmsf and stored on ice
- Add imidazole solution and shake the solution

③ running protein glue

- When adding samples, please see the glue hole, and the gun head should be put into the glue hole and then slowly add samples
- The maker position of different rubber plate is different, which is easy to distinguish
- The amount of maker and protein sample is not the same. Remember to adjust the pipette gun screw in time

Experimental steps

① bacteria:

- 1 The organisms were weighed on a balance and resuspended in 10ml buffer per 1 ㎏
- 2. Before breaking the bacteria, add the protease inhibitor pmsf to the resuspended

bacterial solution, and add 100ul of the inhibitor to every 10ml bacterial solution

- 3. Wash the high-pressure cell cruter with RO water twice, and then twice with buffer to keep the pressure at 300bar-400bar
- 4. Add the bacterial liquid to the instrument and keep the rupture pressure at 900bar1100bar
- 5. Repeat the bacteria 3-4 times, and the bacterial liquid is no longer sticky
- 6. After tetanus, pack with 2ml EP tube, make corresponding marks, and centrifuge at 14000 rpm 10℃ in centrifuge for 30min (leave one tube without centrifuge for sample preparation)
- 7. After centrifugation, keep- - -tube precipitation, - -tube supernatant (sample preparation). The remainder of the supernatant can be collected.

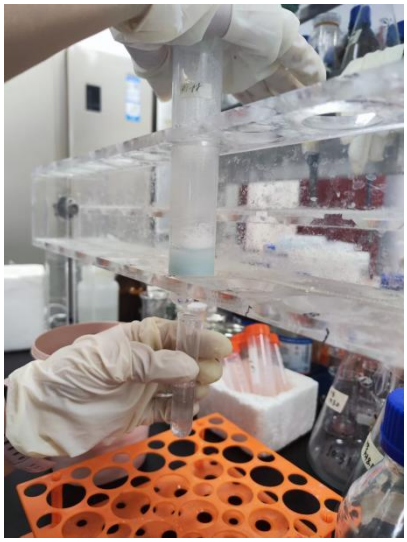
② purification

- 1. Cleaning: Clean the purification column with tap water and RO water with a concentration of 0.1M NaoH immersion purification column 15min washed NaoH from the purified column and washed with RO water, first adding appropriate amount of nickel beads to the purified column and then washed twice with RO water and buffer.
- 2. After washing, the nickel beads were activated by adding 10 mM imidazole to the purified beads for 5min with nickel beads
- 3. After activating the nickel beads, add the collected supernatant night immediately, and mix the sealing membrane sealing tube in the silent mixer for 60min (so that the target protein is fully combined with the nickel beads)
- 4. After mixing, take the penetrating fluid and place it on ice.
- 5. 10 mM imidazole, 30 mM imidazole, 50 mM, 100 mM, 150 mM, 300 mM, and 600 mM imidazole. Each concentration of imidazole should be washed until the eluate meets G250 Coomassie blue.
- 6. Each different concentration of imidazole eluate was collected, with 1u of pmfs protease inhibitor per 1ml eluate and marked in the refrigerator

③ running protein glue

- 1. Make the collected precipitation, supernatant, passage, whole, and each concentration of imidazole eluate.20uloading and 80ul samples were mixed and boiled at 100℃ for 10min
- 2. After sample preparation, the target proteins were tested by SDS-PAGE. During loading, take 10u of each sample and 2ul of protein marker
- 3. After 40-50min of electrophoresis, glue was stained for 10-12h, decolorization and further analysis

Experimental results



Rubber ing, ultrafiltration, A2, F7 activity determination

Thursday,June 29, 2023

Overview

- 1. Protein bands were observed after decigmentation of the glue stained yesterday
- 2. Only ultrafiltration of two protein solutions, fast speed
- 3. The activity was measured after ultrafiltration of A2 and F7. In all the PH of A 2 were measured, with almost no activity, and only one tube was measured in F7

Experimental equipment

① ultrafiltration:

- 1 PBS buffer, G250 Coomassie bright blue, ultrafiltration tube, centrifuge

② determination of activity:

- The protein, the substrate, 1mMNAD +, and 1 PBS buffer were purified

Matters need attention

- 1 . Do not exceed the white scale with the protein fluid or buffer
- .2 When cleaning the inner tube of the ultrafiltration tube, do not rinse directly. Gently pour the water from the outer tube into the cleaning
- .3 Wash the cuvette dish 4 to 5 times each time
- .4 Determine the activity of the enzyme to move quickly

Experimental steps

① ultrafiltration

- 1 . Wash RO water in the ultrafiltration tube, and add RO water and buffer for 5min
- .2 The collected protein solution was added to the ultrafiltration tube, flattened and centrifuged at 3860 rpm 10℃ in a centrifuge for 10 min
- .3 G250 was used to detect the inner and outer tubes respectively. The color of the inner tube

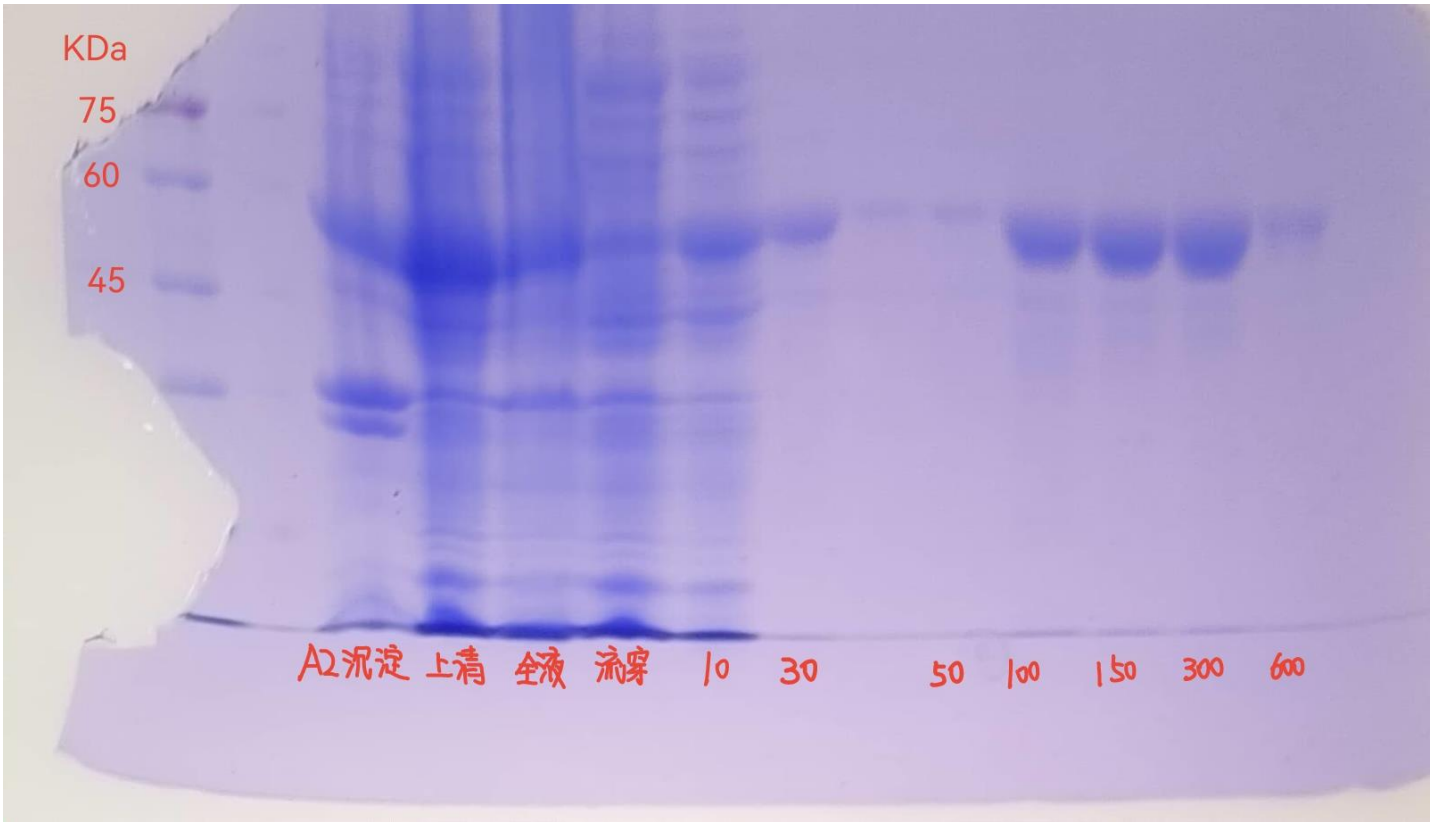
became blue, the color of the outer tube was unchanged, the protein was not leaked from the inner tube, the outer tube liquid was poured, and the corresponding protein liquid was added to the inner tube layer

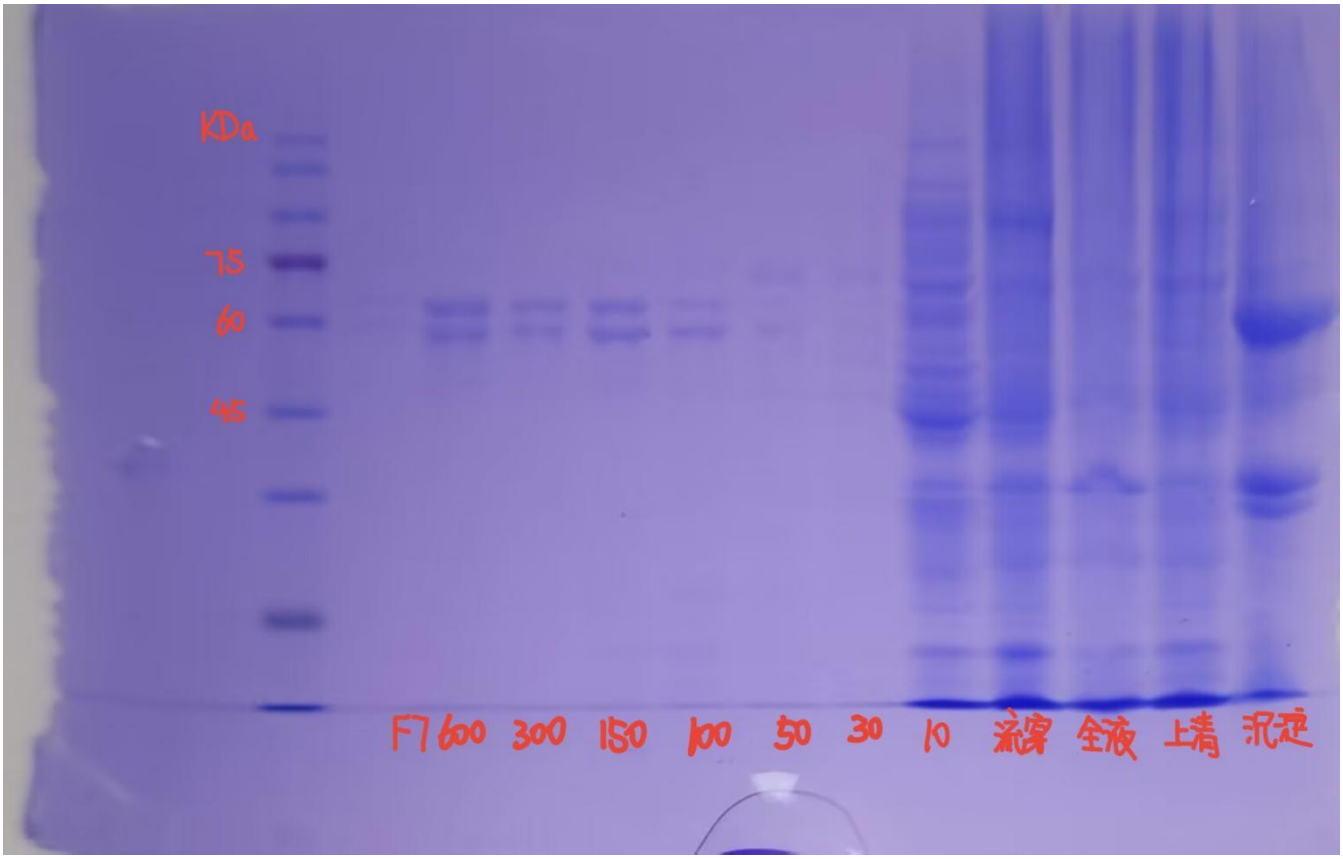
- 4. The protein solution was concentrated, and buffer was added to the ultrafiltration tube for centrifugation and repeated twice, leaving about 500ul of the inner tube
- 5. Use the concentrate in the inner tube 1.5ml EP tubes for 200ul each
- 6 . The packaged proteins were flash-frozen with liquid nitrogen and stored in-80℃ refrigerator. A2 directly measured activity and F7 was frozen tomorrow

② enzyme assay:

- 1 .15ul 1M NAD +, 15ul 1mM of methanol, and 90ul PBS buffer were added to a 1.5-ml EP tube
- 2 . The above solution was transferred to a washed pivette
- .3 Quickly add 30 ul of A 2 protein solution to the cuvette, and quickly press the "measure" button on the computer to start the measurement
- 4. The measurement was stopped after 2min of reaction and the curve was observed

Experimental results




KDa
75
60
45
F7
600
300
150
100
50
30
10
流穿
全液
上清
沉淀

F7 optimum reaction for pH and temperature

Satur day,June 30, 2023

Overview

- Solution reaction conditions of pH of the gradient and temperature were set to explore the optimal reaction pH and temperature of F7

Experimental equipment

- Buffer of 1m MNAD +, 10 mM formaldehyde, different pH
- water-bath

Experimental steps

① Explore the best for pH

- 1. Configure the buffer with pH of 5.97,6.52,6.67,7.06,7.37,7.57,7.88,8.05, and 8.37
- 2. 15ul1MNAD +, 15ul10mM formaldehyde, 90u l pH 5.97 buffer were added in 1.5ml EP tubes
- 3. Transfer the above solution to a pivette and return to zero (find the AUTO-ZERO button on the computer)
- 4. After zero, 30 ul of F 7 protein solution was added to the cuvette. Quickly press the "measure" button on the computer to start the measurement
- 5. Change the buffer to pH solutions of 6.52,6.67,7.06,7.37,7.57,7.88,8.05,8.37, and repeat the above operations
- 6. Record the change range of Abs in the 60s interval for each group (for example, the value of Abs at 60s minus the value of Abs at 0s)

② Explore the optimal temperature

- 7. Add 15ul1MNAD +, 15ul10mM formaldehyde, 8.05 buffer of 90 pH 0 i (pH for the previous step) to 1.5ml EP tube, take another 1.5mlEP tube and add 30 ul of F 7 protein solution to it
- 8. Heat it in a 40 degrees C water bath for 2-3 minutes
- 9. Remove the solution immediately for activity measurement
- 10. Change the temperature of the water bath to 50,60 degrees Celsius and repeat the

above operation

- 11 The range of Abs within the 60s interval was recorded for each group

Experimental result s

① optimum pH:

The change range of Abs within the 60s interval for each group is recorded in the table below:

	A	B	C	D	E	F	G	H	I	J
1	PH	5. 97	6. 52	6. 67	7. 06	7. 37	7. 57	7. 88	8. 05	8. 37
2	Changes in the Abs within 0 - 60s	0. 083	0. 137	0. 175	0. 147	0. 194	0. 254	0. 250	0. 270	0. 204

- C

on

clu

sio

n: The optimal reaction pH of F7 is 8.05

② optimum temperature

The change range of Abs within the 60s interval for each group is recorded in the table below:

	A	B	C	D
1	temperature (℃)	40	50	60
2	Changes in the Abs within 0 - 60s	0. 302	0. 264	0. 220

- Conclusion: The optimum reaction temperature for F7 is 40℃

In conclusion, the optimal reaction conditions for F7 are pH=8.05 and T=